IN VITRO INTERFERENCE OF BACTERIAL QUORUM SENSING AND VIRULENCE FACTORS BY CERTAIN BIOACTIVE NATURAL PRODUCTS

THESIS

SUBMITTED FOR THE AWARD OF THE DEGREE OF

Doctor of Philosophy

IN

AG. MICROBIOLOGY

BY

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Certificate

This is to certify that the work embodied in this thesis entitled "***In vitro* interference of bacterial quorum sensing and virulence factors by certain bioactive natural products**" has been carried out by **Mr. Fohad Mabood Husain** under my supervision. The work included in this thesis is original unless stated otherwise, and has not been submitted for any other Degree. The work is suitable for the award of Ph.D. degree in (Ag.) Microbiology of the Aligarh Muslim University, Aligarh.

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Declaration

I hereby declare that the work embodied in this thesis entitled "***In vitro* interference of bacterial quorum sensing and virulence factors by certain bioactive natural products**" has been carried out by me for the award of Ph. D. degree in (Ag.) Microbiology at the Department of Agricultural Microbiology, Aligarh Muslim University, Aligarh.

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ACKNOWLEDGEMENT

All praises and thanks are for the almighty Allah the One Universal Being, Who enlightened my life with the light of knowledge and guiding me in right direction and showering upon me His blessings for completing my research work.

I feel privileged in expressing my profound sense of gratitude, high esteem and indebtness to my venerable supervisor ***Dr. Iqbal Ahmad****, Associate Professor and Chairman, Department of Agricultural Microbiology, Faculty of Agricultural Sciences, Aligarh Muslim University, Aligarh, for his excellent spirit, illuminative and precious guidance, keen interest, critical opinion and suggestion. He has taught me, both consciously and un-consciously, how good experimental microbiology is done. I appreciate all his contributions of time, ideas, to make my Ph.D. experience productive and stimulating. His constant useful encouragement and sympathetic attitude has always been a source of inspiration during the entire course of preparation and formation of thesis. I can never forget his help and cooperation in my struggle for education. His affectionate behaviour throughout my research programme boosted my morale. I would ever try to follow his footprints in my professional and personal life.*

I take this opportunity to thank the Dean, Faculty of Agriculture Sciences for providing me all the basic requirements to complete my research work at this department.

I am highly thankful to my teachers Prof. Javed Musarrat, Dr. Abdul Malik, Dr. Mohd. Saghir Khan and Dr. Almas Zaidi (D/o Ag. Microbiology) for their helping nature, valuable suggestions and support throughout the research programme.

Due thanks are expressed for Dr. Farrukh Aqil and Dr. Maryam Zahin, Brown Cancer Center, University of Louisville, Kentucky, for the guidance, critical suggestions, constant support and encouragement during my research work.

I wish to express my humble thanks to Shaba Apa for her care as an elder, continuous support and encouragement at each and every step of my research work, leading me to the right path at every stage when needed. I also appreciate the support, guidance and cooperation received from Mr. Fazalur Rahman Khan, the go-to man of the department, who always has something fruitful to offer especially in times of crisis.

I appreciate the contributions received from Mr. Rizwan Salim Kazi, Mrs. Rizwana Khananm and other members of the office staff Mr. Usman, Mr. Sanjay Kumar and Mr. Sultan for always being in the department to help and support me in my work and organizing any programme in the department.

I wish to thank librarian Mrs. Bushra Maqbool for her assistance provided in the seminar library. I'm whole heartedly thankful to Mrs. Darakshan Noor and Mr. Siraj for their cooperation, goodness and timely providing easy access to a plethora of books in the seminar library.

I earnestly wish to make a special mention of Dr. Farhana Masood whose constant cooperation and timely help lead me to overcome my problems. She enlighted me on various aspects of my study, guided me against her valuable time and helped me a lot. Her sustained help throughout my research work can by no means be legibly or verbally expressed.

The idiom "A friend in need is a friend indeed" has somewhat changed for me as I found that "A friend in need is brother indeed". I am lucky to have friends like Javed Bhai, Owais bhai, Zubair, Mashihur, Yusuf, Ees, Zainul and Zeeshan with whom I shared all my happiness and stress. They are my pillars of strength and they have been the ones who always motivated me and strengthened my belief that I could do this.

I will be failing in my duties if I don't acknowledge the support of Dr. Nahid who was more than a doctor for my mother. The care and sincerity she showed as a doctor was just commendable. I will be always indebted to her.

I very sincerely thank Prof. Qudsia Tahseen, Department of Zoology, for allowing me use to her lab and my dear friend Mr. Mohammad Asif for helping me perform the experiments on C. elegans.

For this dissertation I'm grateful to Prof. Soumyo Mukherji, Head, Sophisticated Analytical Instrument Facility, Indian Institute of Technology, Bombay for allowing gas chromatography-mass spectrometry analysis of my sample. Dr. S. Vijayalakshmi, Research Scientist at SAIF, IITB is appreciated for her cooperation. I also acknowledge technical support received from Dr. Ruchita Pal, Mr. Ajay Kumar and Mr. Plabon Bohra at Advanced Instrumentation and Research Facility, Jawaharlal Nehru University, New Delhi, during Scanning electron microscopy, gas chromatography-mass spectrometry and Mass spectroscopy experiments. I am very grateful to Dr. S Farooq, Director, Himalaya Drug Company, Dehradun, for allowing me to perform HPTLC analysis of my plant samples. Thanks are also due to Dr. Zafar Mehmood for extending his kind cooperation in carrying out HPTLC analysis. I would also like to thank Macrogen Incorporation, Seoul, South Korea for 16s rDNA gene sequencing of our test strains. For physico-chemical analysis of essential oils, I highly appreciate co-operation of Mr. K. N. Dwivedi, Principal Director and Mr. Nadim Akbar, Assistant Director at Fragrance and Flavour Development Centre, Kannauj. I am also thankful to Mr. Afzal, Department of Chemistry, AMU, Aligarh for IR analysis of my plant samples.

I gratefully acknowledge University Grants Commission and Department of Science and Technology, Ministry of Science and Technology, New Delhi for awarding me fellowship. This financial assistance has truly helped me to make my Ph.D. work possible.

I express my thanks to my seniors Dr. Farah Ahmad, Dr. Ikram Ansari, Dr. Mohd. Zubair Alam, Dr. P.A. Wani, Dr. Munees Ahmad, Dr. Sourabh

Diwedi, Dr. Qaiser Saquib and Dr. Reshma Anjum who helped me to overcome the difficulties in the initial stages of my research work.

I'm thankful to all my lab mates at the research laboratory in the department of Agricultural Microbiology in particular Musheer Altaf, Shoukat, Meenu, Samira, Sana, Saima for their time and ideas spent with me. My reserved thanks are due to Shavez for his help and support as younger brother and especially at the time of compilation of this thesis. I am also thankful to all these research scholars for encouraging me towards my target of research. I acknowledge all the love and support received from my juniors and students of M.Sc. (Microbiology) during my Ph.D. programme.

My time at AMU was made enjoyable and conducive in large part due to the many friends that became a part of my life. I am grateful for memorable trips, entertainment and time spent with Qasim, Shakeela, Jamal, Mohsin, Sumit, Asim, Arif, Asim Masood, Saad, Iqbal, Hridesh, Kamran, Atif, Husain, Farhan and many more, the list is not exhaustive. They were always with me and inspired me in research and life through our interactions during the long hours outdoor the lab.

Memories goes back to remember and thank my childhood friends Musharraf, Sufiyan, Shajar, Abhinav, Akhilesh, Anant, Saddam, Nishant and Tahseen for being backbone of my friendship. Their support and encouragement in the different phases of my study and personal life is greatly appreciated. Whenever I need them they stood behind me.

My deepest gratitude goes to my family for their unflagging love and support throughout my life. I'm indebted to my parents for their care and love. They sacrificed everything to give me the best possible education. I am highly obliged to my elder brother Mr. Arshad Mahmood for his moral support and encouragement throughout my education period. He spared no effort to provide me the best possible environment financially and morally to grow up and to complete my doctoral degree. My aunt (phuphi), Nafis fatima, is no longer with us, but she is forever remembered and loved. I remember her constant support whenever I encountered difficulties in my life. I have no suitable words that can fully describe her everlasting warmth and love to me that I still feel. I thank my family from the bottom of my heart for the encouragement and well wishes. I'm grateful for affection, attention and encouragement received from my brothers and sisters. I must thank my nephews and niece for being so affectionate, after the hard day's work they were the stress busters.

Last but not least, thanks are due for many other people and memories that I could not mention here but are precious part of my life and have always been well wishers of mine in one or other form.

(Fohad Mabood Husain)

Dedication

This thesis is dedicated to my beloved parents for their endless love, support and encouragement

And

Sir Syed Ahmad Khan: the Pioneer of Humanism

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Abbreviations used in the study

AHL	Acylhomoserine lactone
AI	Autoinducer
β	Beta
CLSI	Clinical and Laboratory Standards institute
CV	*Chromobacterium violaceum*
DMSO	Dimethyl sulfoxide
et al	et alia
ECR	Elastin congo red
EDTA	Ethylene diamine tetraacetic acid
EPS	Exopolysaccharide
°C	Degree celsius
GC	Gas chromatography
GC-MS	Gas chromatography–mass spectrometry
h	Hour
HPTLC	High pressure Thin layer chromatography
HSL	Homoserine lactone
IR	Infra red
LB	Luria-Bertani
µg	Microgram
µl	Microliter
ml	Milliliter
mm	Millimeter
mM	Millimolar
MIC	Minimum inhibitory concentration
MS	Mass spectrometry
m/z	Mass-to-charge ratio
nm	Nanometer
C12-HSL	N-(3-oxododecanoyl)-L-homoserine lactone
C4-HSL	N-butanoyl-L-homoserine lactone
C6-HSL	N-hexanoyl-L-homoserine lactone
OD	Optical density
ONPG	Ortho-nitrophenyl-β-D-galactopyranoside

PBS	Phosphate buffer saline
QS	Quorum sensing
QSI	Quorum sensing inhibitor
SDS	Sodium dodecyl sulphate
SEM	Scanning electron microscopy
TCA	Trichloroacetic acid
UPLC	Ultra performance liquid chromatography
UV	Ultraviolet
v/v	Volume/volume
X-gal	5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside

Infectious diseases are still one of the major causes of morbidity and mortality of human across the globe. Emergence of many new pathogenic microorganisms and re-emergence of old microbial threats have continued to challenge public health and infectious disease research communities' worldwide (Fauci et al. 2005). Antibiotics have proven to be powerful drugs for the control of infectious diseases and remain one of the most significant discoveries in modern medicine. Their extensive and unrestricted use has, however, imposed a selective pressure upon bacteria, leading to the development of antimicrobial resistance (Russel, 2003; Oluwatuyi et al. 2004; Schelz et al. 2010). The capacity of bacteria to acquire and transmit genetic determinants of resistance is a conserved evolution strategy and has exacerbated the worldwide resistance problem. Antibiotic resistance is recognized by the World Health Organization (WHO) as the greatest threat in the treatment of infectious diseases (Walker and Levy, 2001; Wright, 2005). WHO has promoted a complex action plan, based on the slogan "No action today, no cure tomorrow" that includes strategic actions for mitigation, prevention and control (WHO, 2011). Drugs recently developed to combat emerging antibiotic resistances, such as resistance to vancomycin, linezolid, and the latest beta-lactams, have themselves already lost effectiveness against some bacterial strains (Arias and Murray, 2008; Yong et al. 2009; Applebaum, 2012).

Even more discouraging, development of new drug leads has slowed dramatically over the past 10 years, and newer drugs that have been successfully developed are strictly reserved to treat only the most serious infections, so as not to repeat over usage mistakes of the past (Dellit et al. 2007). Further, increased antibiotic resistance has led to the introduction of combination therapy which has increased treatment efficacy and contained drug resistance to some extent (Athamna et al. 2005). Although combination therapy provided the answer to antibiotic resistance for a while, there have been reports of emerging resistance to drugs in combination and multi-drug resistance in common pathogenic bacteria (so called "superbugs") (Rodas-Suárez et al. 2006). The major group of problematic MDR bacteria includes *Mycobacterium tuberculosis*, methicillin resistant *Staphylococcus aureus* (MRSA), Vancomycin resistant *E. coli* (VREC), *Pseudomonas aeruginosa* and ESβL producing MDR entric bacteria and others (Ahmad et al. 2009).

Therefore, there is a critical need to discover new antimicrobial compounds and to identify new methods for disease prevention and treatment (WHO, 2011). It is therefore more important than ever to develop therapies that will provide sustainable, long-term effectiveness against bacterial pathogens. Compounds that aim at killing or inhibiting growth of micro organisms have been applied in the process of preserving food, preventing biofilm growth and surface fouling and particularly in the treatment of bacterial infections. This strategy has an obvious drawback: when growth of bacteria is blocked, the bacteria are under harsh selective pressure to develop resistance (Rasmussen and Givskov, 2006). This calls for development of alternative treatment strategies which rely on identification of a suitable drug target and development of cognate pharmaceutically applicable drugs.

One appealing approach, is to inhibit the virulence of pathogenic bacteria which are under the control of a global regulatory mechanism called quorum sensing (QS) (Hentzer et al. 2003). Quorum sensing (QS) is a widespread phenomenon which facilitates the coordinated regulation of gene expression in bacteria as a function of cell population density (Winans and Bassler, 2002). In Gram-negative bacteria, the most intensively investigated QS systems employ *N*-acyl homoserine lactone (AHL) signal molecules (Wu et al. 2004). At low population densities, AHLs are present at a basal concentration level. As the population density increases, the accumulated AHLs reach concentrations that allow binding the regulators, and the complexes then activated or inactivated and implicated in pathogen and host interactions, such as antibiotic production and the expression of virulence factors (Sircili et al. 2004). In different bacterial genera, these may include genes which code for the production of secondary metabolites, plasmid transfer, motility, virulence, and biofilm development (Williams, 2007). Since QS controls a range of biological functions associated with virulence and pathogenicity and biofilm formation, interrupting QS may represent one such method which has the added advantage that the targets are not normally essential for bacterial survival and therefore are not subject to the same selective pressures observed for conventional growth inhibitory antimicrobials (Bjarnsholt and Givskov, 2008; Lowery et al. 2010). By doing so, the infecting bacteria may fail to adapt to the host environment and establish an infection. For some bacteria, working together as a group provides a means to build a defense or to surmount a barrier that individual bacterial cells find impossible to achieve.

Blocking interactions between bacteria would effectively force bacteria to live as individuals fending for themselves (Rasmussen and Givskov, 2006). Newer strategies have sought to target components of bacteria that are responsible for pathogenesis rather than targeting components that are essential for growth and, as such, have garnered the name "antivirulence" or "antipathogenesis" therapies (LaSarre and Federle, 2013).

There are three different targets in gram negative bacterial QS system such as: (a) the signal generator (b) the signal molecule (c) the signal receptor.The present strategy for detecting and developing anti QS anti-infective from natural products including microorganism includes (a) bacterial metabolites and natural products that prevent the signal molecule from being synthesized by the luxI encoded AHL synthase. (b) Signal molecule degrading enzyme or conditions for inactivating the signal molecules. (c) Small molecule with variations in their chemical composition that would allow them to block the AHL receptors. The various derivatives of AHL analogue may act as agonist or antagonist (Rasmussen and Givskov, 2006; Kalia, 2013).

Pseudomonas aeruginosa is a highly successful opportunistic pathogen that displays intrinsic multidrug resistance and has a tremendous capacity to acquire further resistance mechanisms. It is known to cause nosocomial infections, blood stream infections, Pneumonia and burn wound infections. It is also associated with chronic infections of the respiratory pathways including cystic fibrosis, diffused panbronchitis, and chronic obstructive pulmonary diseases (Adonizio et al. 2008a). During chronic infection, the bacterium can form a protective biofilm therefore reducing the efficacy of existing antibiotics. *P. aeruginosa* also harbors an impressive range of virulence factors, many of which are controlled by the quorum-sensing system (Fothergill et al. 2012). The *P. aeruginosa* QS circuits make attractive targets for novel antimicrobials because QS controls virulence factor production and no homologs to known QS components exist in humans. This is especially critical in the treatment of persistent infections in cystic fibrosis patients given the resistance of many *P. aeruginosa* isolates to available antibiotics (Mattmann and Blackwell, 2010). *P. aeruginosa* harbors three QS systems: two LuxI/LuxR-type QS circuits that function in series to control expression of virulence factors as well as a third, non-

LuxI/LuxR-type system called the *Pseudomonas* quinolone signal (PQS) system (Rutherford and Bassler, 2012). *P. aeruginosa* QS-activated virulence factors include elastase, proteases, pyocyanin, lectin, swarming motility, rhamnolipids, and toxins (Dekimpe and Deziel, 2009). QS mediated biofilm formation in *P. aeruginosa* and other pathogens is the major cause of persistent infections. Biofilms are complex aggregation of microorganisms encased in a self secreted exopolymeric matrix consisting of EPS (Costerton et al.1995). Centers for Disease Control and Prevention, USA, states that 65% of all infections are caused by biofilms (Lewis, 2007). It has also been found that bacteria living in the biofilm mode of growth are often up to 1000 times more resistant to antibiotic than their planktonic counterparts as the biofilm matrix conserves degraded bacteria and nutrients, and it affords superior resistance to antimicrobial agents, dehydration, UV, and other environmental stressors. (Caraher et al. 2007).

The first Quorum sensing inhibitory activity was characterized in a seaweed *Delisea pulchra* (Rasmussen et al. 2000). Since then numerous compounds have been reported to inhibit QS, a few have, with great success been tested in animal models. Unfortunately, these compounds are unsuitable for human use. The halogenated furanones are unstable and the fungal compounds identified so far are mycotoxins (Rasmussen and Givslov, 2006). Therefore, we need to search for other safe and stable agents. The plant kingdom has long been a source of medicines, and as such, there have been many ethnobotanically directed searches for agents that can be used to treat infection.

During the last few years, medicinal plants have attracted the attention of pharmaceutical and scientific communities, and evidence has demonstrated the promising potential of antimicrobial plant-derived substances (Cowan, 1999; Verpoorte et al. 2005; Savoia, 2013). The antimicrobial effect of plant essential oils and extracts has formed the basis of many applications, including raw and processed food preservation, pharmaceuticals, alternative medicine and natural therapies. Moreover, plant extracts/compounds are widely accepted due to the perception that they are safe and they have a long history of use in folk medicine as immune boosters and for the prevention and treatment of several diseases (Ahmad et al. 2006; Eloff et al. 2011).

Over the years, the use of medicinal plants, which forms the backbone of traditional medicine, has grown with an estimated 80% of the populations, mostly in developing countries, relying on traditional medicines for their primary health care (Ahmad et al. 2006; WHO, 2011, 2012). Plants are rich in several secondary metabolites and are a major source of chemical diversity; therefore, they are a potential source of new drugs for man whose use to control diseases is a centuries-old practice. Among 109 new antibacterial drugs, approved in the period 1981–2006, 69% originated from natural products (Newman, 2008). Modern science and technological advances are accelerating the discovery and development of innovative pharmaceuticals with improved therapeutic activity and reduced side-effects from plants. Plant-derived substances under intensive research for possible applications include crude extracts of leaves, roots, stems and individual compounds isolated from these plants/parts, essential oils and essential oil components.

In the recent years an increased interest has been shown by the scientific community to screen and search anti-QS activity from natural products as reviewed by many workers across the globe (Kociolek, 2009, Kalia, 2013; Husain and Ahmad, 2013). Some authors such as Yang et al. (2009) have attempted computer aided identification of recognized drugs as QS inhibitors. They have suggested that structure based virtual screening is an efficient tool in search of novel compounds. Quorum sensing inhibitors have also been reported in various natural products including medicinal plant species from South Florida (Adonizio et al. 2006; 2008a), in fruits and spices (Vattem et al. 2007, Abraham et al. 2012) and phytocompounds (Vandeputte et al. 2011). Essential oils possessing anti-QS property have also been reported by many workers (Szabo et al. 2010; Jaramillo-Colorado et al. 2012). However, rich diversity of Indian medicinal plants has not been yet screened for their quorum sensing inhibitory properties. Only few reports of preliminary nature are available (Musthafa et al. 2010; Abraham et al. 2012). We have previously screened a number of Indian medicinal plants for antibacterial (Ahmad et al. 1998; Ahmad and Beg, 2001), antifungal (Aqil and Ahmad, 2003), antioxidant (Aqil et al. 2006) and antimutagenic activities (Zahin et al. 2010a). Considering the rich diversity of traditionally used medicinal plants and essential oil and their uses in a number of ailments/infectious diseases in traditional system of medicine, it is expected that systemic screening and evaluation of Indian medicinal plants will hopefully provide

new bioactivity relevant to anti-pathogenic drug principle. In the present study we have selected a variety of natural products including antibiotics, medicinal plants and common essential oils in search of promising agents interfering with quorum sensing and virulence factors in Gram-negative bacteria specially *Chromobacterium violaceum* and *Pseudomonas aeruginosa* strains. Majority of the natural products selected are not previously screened for anti-QS activity, however, they are known for other biological activities.

Therefore, screening of plant and its derived compounds may facilitate the discovery of compounds that attenuate bacterial pathogenesis by interfering with QS systems and render pathogenic bacteria non-virulent without affecting their viability. This will generate less pressure for the evolution of resistance as compared to antibiotic therapy. These plants and plant products can offer a large and attractive repertoire for the discovery of quorum sensing inhibitors. Therefore, the present study is planned with the following aims and objectives:

Objectives:

1. Isolation, characterization and detection of quorum sensing signal molecules in *Pseudomonas aeruginosa* and other Gram negative bacteria.
2. Screening of natural products (antibiotics, plant extracts and essential oils) for their quorum sensing interference activity against *Chromobacterium violaceum* biosensor strains.
3. Evaluation of active anti-QS antibiotics on quorum sensing regulated virulence factors and biofilm formation in the strains of *Pseudomonas aeruginosa* and *Aeromonas hydrophila*.
4. Fraction based activity of active plant extracts and active essential oils on quorum sensing regulated virulence factors and biofilm of test bacterial strains.
5. To evaluate the therapeutic potential of active extracts, essential oils and compounds in *Caenorhabditis elegans* nematode model.
6. Phytochemical analysis of selected plant extracts and essential oils.

Review of literature

2.1. Medicinal plants in health care: An overview

Plants have formed the basis of traditional systems of medicine that have been in existence for thousands of years and continue to provide mankind with new remedies. The use of natural products with therapeutic properties is as ancient as human civilization and for a long time, mineral, plant and animal products were the main source of drugs (de Pasquale, 1984). For centuries, people have used plants for healing. Plant products as part of food or botanical portions and powder have been used with varying success to cure and prevent diseases throughout history (Raskin et al. 2002). Ethnomedicinal plants are used both for primary health care and for treating chronic diseases such as AIDS, cancer, hepatitis disorders, heart and old age related diseases like memory loss, osteoporosis and diabetic wound. The Indian coded system includes, Ayurveda, Unani, Siddha and Amchi (Krishna, 2003). The Ayurvedic concept appeared and developed between 2500 and 500 BC in India (Subhose et al. 2005).The literal meaning of Ayurveda is "Science of life," because ancient Indian system of health care focused on views of man and his illness. It offers programs to rejuvenate the body through diet and nutrition. It offers treatment methods to cure many common diseases such as food allergies, which have few modern treatments (Gurib-Fakim, 2006).

The biogeographic position of India is unique as we have diverse ecosystem, ranging from the humid tropics of Western Ghats to the Alpine Zone of Himalaya and from the dry deserts of Rajasthan to the tidal mangroves of the Sunderbans and hence India is endowed with a rich flora (Dahanukar and Hazra, 1995; Zahin et al. 2010a). India is the largest producer of medicinal plants. In India, around 20,000 medicinal plants have been recorded; however, traditional practitioners use only 7,000–7,500 plants for curing different diseases. In India, around 25,000 effective plant-based formulations are used in traditional and folk medicine. More than 1.5 million practitioners are using the traditional medicinal system for health care in India. It is estimated that more than 7800 manufacturing units are involved in the production of natural health products and traditional plant-based formulations in India, which requires more than 2000 tons of medicinal plant raw material annually (Pandey, 2008). More than 1500 herbals are sold as dietary supplements or ethnic traditional medicines (Patwardhan et al. 2005)

In Indian systems of medicines, use of different plants for treating ailments was based on the fact that the additive or synergistic effects of the secondary metabolites present in those plants enhance therapeutic viability of the phytoconstituents. This knowledge and experiential database can provide new functional leads to reduce time, money and toxicity – the three main hurdles in drug development. These records are particularly valuable, since effectively these medicines have been tested for thousands of years on people. Efforts are underway to establish pharmacoepidemiological evidence base regarding safety and practice of Ayurvedic medicines. Randomized controlled clinical trials for rheumatoid and osteoarthritis, hepatoprotectives, hypolipedemic agents, asthma, Parkinson's disease and many other disorders have reasonably established clinical efficacy (Mukherjee et al. 2010).

The successful introduction of these plants into modern therapeutics indicates that other discoveries are waiting to be made. The plants used in Indian Systems of Medicine showed the presence of a variety of chemical entities, belonging to different classes. Combining the strengths of the knowledge base of complementary alternative medicines like Ayurveda with the dramatic power of combinatorial sciences and High Throughput Screening (HTS) will help in the generation of structure-activity libraries (Cordell and Colvard, 2005). The development of drugs from ethnic plants continues, with drug companies engaged in large scale pharmacologic screening of herbs (Gold, 2000). There is a revival of interest in herbal products at a global level; herbs such as turmeric, neem, ginger, holi basil and ashwagandha are a few examples of what is gaining popularity among modern physicians.

In the last few decades enormous amount of literature/research data has been published showing diversity of bioactivities of medicinal plants and their phytocompounds/formulations. Findings have shown their useful properties like anticancer, antitumour, antimutagenic, antioxidant, hepato-protective, antiviral, antimalarial, antidysenteric, antiseptic, antistress and immunotherapeutic, antibacterial, antifungal and several other pharmacological actions (Cowan, 1999; Tiwari, 2004; Musarrat et al. 2006; Nostro, 2006; Heinrich, 2010, Savoia, 2013; Leonti and Casu, 2013). Natural products derived from plant sources have contributed significantly in drug discovery. Examples include artemisinin, atropine, digoxin,

ephedrine, gallanthamine, morphine, physostigmine, quinine, reserpine, salicylic acid, sennoside, Taxol, vincristine, vinblastine, glycyrrhizin, and psoralen (Tiwari, 2004; Ahmad et al. 2006; Mukherjee et al. 2010).

The problem of MDR in bacterial diseases has become global and search for novel bioactive compounds which can prevent infection by reducing pathogenicity and virulence of bacteria is one potential target to combat such problematic organisms. In the last decade novel bioactivities of medicinal plants extracts, phytocompounds and other natural products have been documented such as antibiofilm, anti-resistance, anti-virulence etc. One of the recent activities which have now gained momentum is the plant and plant product influencing cell to cell communication of bacteria-which is described as Quorum sensing (Bauer and Tepletski, 2001; Schauder and Bassler, 2001). Breakdown of this system causes attenuation of microbial pathogenicity (Smith and Iglewski, 2003; Wu et al. 2004; Ahmad et al. 2011; Kalia, 2013). The discovery of anti-quorum sensing agents in plants provides us with yet another type of "antimicrobial" agents to be exploited as an anti-infective agent in near future.

2.2. Ethnobotany of selected plants used in the study

A brief introduction of selected medicinal plants/essential oil used in this study after general screening are given below:

2.2.1. *Mangifera indica* L.

Mangifera indica , also known as mango, belongs to genus *Mangifera* which consists of about 30 species of tropical fruiting trees in the flowering plant family Anacardiaceae. In traditional system of Indian medicine, varied medicinal properties are attributed to different parts of mango tree (Chopra et al. 1992).

Mangifera indica fruit is one of the important tropical fruits in India and contributes major part of the world production. Polyphenolics, flavonoids and triterpenoids are the chief chemical constituents present in the plant. Mangiferin, a xanthone glycoside is the major bio-active constituent, isomangiferin, tannins & gallic acid derivatives. The bark is reported to contain protocatechic acid, catechin, mangiferin, alanine, glycine, γ-aminobutyric acid, kinic acid, shikimic acid and the tetracyclic triterpenoids cycloart-24-en-3β,26diol, 3-ketodammar-24 (*E*)-en-20S,26-

diol, C-24 epimers of cycloart-25 en 3β,24,27-triol and cycloartan-3β,24,27-triol.(Scartezzini and Speroni, 2000)

Most parts of the tree are used medicinally and the bark also contains tannins, which are used for the purpose of dyeing (Chopra et al. 1992; Shah et al. 2010). Traditionally, various parts of plant are used as a dentrifrice, antiseptic, astringent, diaphoretic, stomachic, vermifuge, tonic, laxative and diuretic and to treat diarrhea, dysentery, anaemia, asthma, bronchitis, cough, hypertension, insomnia, rheumatism, toothache, leucorrhoea, haemorrhage and piles. All parts are used to treat abscesses, broken horn, rabid dog or jackal bite, tumour, snakebite, stings, datura poisoning, heat stroke, miscarriage, anthrax, blisters, wounds in the mouth, tympanitis, colic, diarrhea, glossitis, indigestion, bacillosis, bloody dysentery, liver disorders, excessive urination, tetanus and asthma (Shah et al. 2010).

2.2.2. *Psoralea corylifolia* L.

Psoralea corylifolia (Fabaceae), is an erect annual herb widely used in Ayurvedic medicine as well as in Traditional Chinese medicine almost throughout India (Sharma and Dravyaguna, 1986). It is found as a common weed of winter season distributed throughout India in Himalayas, Dehra Dun, Oudh, Bundelkhand, Bengal, Bombay, some valley in Bihar, Deccan, Karnataka and in various plains of India, especially semi-arid regions of Rajasthan and Eastern districts of Punjab, adjoining the Uttar Pradesh (Sharma et al. 2001).

The plant has been widely used in Ayurvedic and Chinese medicine system as a cardiac tonic, vasodilator, pigmentor, antitumor, antibacterial, cytotoxic and antihelmenthic (Khusboo et al. 2009; Gidwani et al. 2011; Purkayastha and Dahiya, 2012). The seeds of the plant possess greater medicinal value and are used in indigenous system of medicine as laxative, aphrodisiac, anthelminitic, diuretic and diaphoretic in febrile conditions. Oral administration and local external application of seeds in form of ointment or paste have been valued in Ayurveda and thus recommended in the treatment for leucoderma, leprosy, psoriasis, hair loss and inflammatory diseases of the skin such as eczema (Gidwani et al. 2011). The seed oil is highly beneficial externally in numerous skin ailments and recommended orally with beatlenut leaf in leprosy. Seeds are helpful in production of perfumes and are effective in bilious disorders; scorpion-sting and snake bite (Panda, 2000; Kapoor,

2001). Roots of the plant are useful in dental caries; fruits are laxative and aphrodisiac in nature. Leaves are good for the treatment of diarrhea (Purkayastha and Dahiya, 2012).

Major phytoconstituents of the plant includes volatile oil, coumarins, flavones, monoterpenoids phenols, chalcones, lipids, resins and stigmasteroids. Phytochemical study of *P. corylifolia* reveals that the nature and amount of phytochemical vary according to climatic conditions. Researchers found that the seeds contain more active constituent than any other parts of plant. Psoralen, isopsoralen, corylifolin, corylin and psoralidin have been isolated from the petroleum ether and chloroform extract of the whole plant of *P. corylifolia* (Jiangning et al. 2005). A monoterpene phenol, bakuchiol (Dev et al. 1973; Jiangning et al. 2005) and the two novel dimericmonoterpenoids, bisbakuchiols A and B, (Cheng et al. 2007) have been isolated from the seeds of *P. corylifolia.* The ethereal seed extract showed the presence of Corylinal (Dhar et al. 1978a) as well as C-formylated chalcone and Isoneobavachalcone (Dhar et al. 1980). Psoralenol, (Dhar et al. 1978b) a new isoflavone has been isolated from seeds of *P. corylifolia.* Psoralen and isopsoralen were separated from *P. corylifolia* by high-speed counter-current chromatography (Liua et al. 2004). Five novel compounds, psoracorylifols A–E (Yin et al. 2006) as well as chalcone and Bavachromanol (Suri et al. 1980) have been reported from seed samples. Four flavonoids bavachinin, bavachin, isobavachin and isobavachalcone were isolated from the seeds of *P. corylifolia* (Haraguchi et al. 2002). Psoralenoside and Isopsoralenoside (Qiao et al. 2006), two new benzofuran glycosides were also isolated. Bavachinin—a flavanone isolated from seeds of *P. corylifolia* has been reported (Anand et al. 1978). The insoluble portion of the ethanol extract of *P. corylifolia* yields neobavachalcone, bavachromene and 7-methyl bavachin (Sood et al. 1977). A new compound, cyclobakuchiol C (Yin et al. 2007) was isolated from the non-polar fraction of the seeds of *P. corylifolia.* Recently, Corylidin, 8-methoxy psoralen, psoralidin-2′, 3′-oxide, corylin, glucose, corylifolinin, neobavaisoflavone, stigmasterol, daucosterol, triacontane, β-sitosterol D-glucoside and saponins have also been isolated from the *P. corylifolia* (Chopra et al. 2013).

2.2.3. *Trigonella foenumgraceum* L.

The fenugreek *(Trigonella foenumgracum* L.*)* is a self pollinating annual leguminous bean which belongs to *Fabaceae* family (Balch, 2003). It is one of the most ancient medicinal herbs (Thomas et al. 2011).

Fenugreek contains different alkaloids, flavonoids and saponins (Uemura et al. 2011; Kumar et al. 2012) but out of all these, saponins are found to be in maximum concentration in the fenugreek (Singh and Garg, 2006). Alkaloid is natural bases containing at least one nitrogen atom in its heterocyclic ring and is found in plants. Alkaloid and volatiles of fenugreek seed are two major constituents which causes bitter taste and bad odour due to which people try to avoid consumption of fenugreek seed and its products (Faeste et al. 2009). Fenugreek contains 35% alkaloids, primarily trigonelline (Ruby et al. 2005), whereas saponin was found to be 4.8% (Rao et al. 1996; Jani et al. 2009). One hundred gram of Fenugreek endosperm is reported to be containing 4.63 g saponin. The alkaloids, flavonoids and saponins of fenugreek have pharmacological effect. They act as antilipidemic, hypoglycaemic and cholagogic agent and their use should be promoted to manage diabetes mellitus, hypercholesterolemia because clinical evidence shows promising results in reducing serum cholesterol level. At the same time, care should be taken to avoid minor gastrointestinal symptoms and allergic reactions on its consumption (Izzo et al. 2005). Murlidhara et al. (1999) however, considered alkaloid and saponins as anti-nutritional factors in human food though the extract of fenugreek containing saponins is found to be enhancing hunger, reducing plasma cholesterol level and hypocholesterolemia in rats (Petit et al. 1995).

Fenugreek is a promising protective medicinal herb for complementary therapy in cancer patients under chemotherapeutic interventions (Naidu et al. 2011). It is reported that fenugreek seeds have lowered serum cholesterol, triglyceride and low-density lipoprotein in hypercholesterolemia suffering patients and experimental models (Singhal et al. 1982; Basch et al. 2003). Fenugreek seed improves peripheral glucose utilization, contributing to improvement in glucose tolerance and exerts its hypoglycemic effect by acting at the insulin receptor level as well as at the gastrointestinal level (Singh and Garg, 2006). Fenugreek contains phenolic and flavonoid compounds which help to enhance its antioxidant capacity (Dixit et al.

2005). Fenugreek has been well known to be used as antidiabetic remedy for both type I and II diabetes and can be an effective supportive therapy in the management of diabetes (Senthil et al. 2010; Das et al. 2011). Besides this Fenugreek is also known to have immunomodulatory effects, antifertility effects, antiulcer properties (Meghwal and Goswami, 2012).

2.2.4. *Syzygium aromaticum* L. (clove) oil

Cloves are the pink flowering bud of a form evergreen tree (*Eugenia aromatica*), which are dried until brown and used for medicinal and spicing purposes. Indigenous to the Moluccas spice islands of Indonesia, cloves also grow naturally in India, the West Indies, Tanzania, Sri Lanka, Brazil and Madagascar. With their sultry sweet aromatic flavor and powerful essential oil compounds, cloves have been used for hundreds of years as a nutritional spice for food and a remedy for a variety of health concerns. For over 2,000 years, both Indian and Chinese traditional medicine made extensive use of clove flowers and clove oil. Arabic traders brought the buds to Europe in 4th century A.D., and in the seventh and eighth century A.D. Europe, cloves became very popular as a medicinal flower, due to their ability to preserve foods, and mask the smell of poorly-kept foods (Bhowmick et al. 2012).

The major component of clove oil is usually considered to be eugenol, with β-caryophyllene and lesser amounts of other components such as benzyl alcohol, but the proportions vary widely (Chaeib et al. 2007). Some of the major components include eugenol (88.58%), eugenyl acetate (5.62%), β-caryophyllene (1.39%), 2-heptanone (0.93%), ethyl hexanoate (0.66%), humulenol (0.27%), α-humulene (0.19%), calacorene (0.11%) and calamenene (0.10%) as reported by Lee and Shibamoto, 2002; Prashar et al. 2006; Pawar and Thaker, 2006.

Clove oil is known to possess antibacterial, antifungal and antiviral properties (Chaeib et al. 2007). The major constituent of clove oil is eugenol, to which are attributed many of the antioxidant properties (Ogata et al. 2000). Jirovetz et al. (2006) found that the antioxidant action of 0.005% clove oil was identical to that of standard butylated hydroxytoluene at a concentration of 0.01%. It can therefore, be used as an easily accessible source of natural antioxidants and in pharmaceutical applications (Gulçin et al. 2004). Clove essential oil has been reported to show anticarcinogenic (Zheng et al. 1992) and antimutagenic potential (Miyazawa and Hisama, 2001; Aisha

et al. 2012). Volatile oils display cytotoxic action towards the human tumor cell lines PC-3 and Hep G2 (Ogunwande et al. 2005; Yoo et al. 2005). Eugenol is used in a wide range of applications, such as a local anaesthetic in dentistry and as an ingredient in dental cement for temporary fillings (Markowitz et al. 1992). It is relatively user-friendly and can be used in lower concentrations than other local anaesthetics (Keene et al. 1998). Clove oil has also been reported for insecticidal activities and it has been shown to inhibit the emergence of *Culex pipiens* larvae (El Hag et al. 1999) and to display insecticidal activity against *Pediculus capitis* (Yang et al. 2003), *Anopheles dirus* mosquitoes (Trongtokit et al. 2005; Phasomkusolsil and Soonwera, 2011).

2.2.5. *Mentha piperita* L. (peppermint) oil

Peppermint (*Mentha piperita* L.) is a perennial herb native to Europe, naturalized in the northern USA and Canada, and cultivated in many parts of the world. A hybrid of spearmint (*M. spicata* L.) and water mint (*M. aquatica* L.), peppermint grows particularly well in areas with high water-holding capacity soil. Best known for its flavoring and fragrance properties, peppermint leaves (fresh and dried) and the essential oil extracted from the leaves are used in many foods, cosmetic and pharmaceutical products (McKay and Blumberg, 2006).

The chemical components of peppermint leaves and oil vary with plant maturity, variety, geographical region and processing conditions (Maffei and Scannerini, 1992; Rohloff, 1999; Blanco et al. 2002; Xu et al. 2003). The fatty acid composition of the non-polar lipid fraction of peppermint leaves is dominated by palmitic (16:0), linoleic (18:2) and linolenic (18:3) acids (Maffei and Scannerini, 1992). The main volatile components identified in the essential oil of peppermint are menthol (33–60%), menthone (15–32%), isomenthone (2–8%), 1,8-cineole (eucalyptol) (5–13%), menthyl acetate (2–11%), menthofuran (1–10%), limonene (1–7%), β-myrcene (0.1–1.7%), β-caryophyllene (2–4%), pulegone (0.5–1.6%) and carvone (1%) (Clark and Menary, 1981; Sang, 1982; Pittler and Ernst, 1998; Dimandja et al. 2000; Gherman et al. 2000).

The antioxidant capacity of peppermint has been determined using a number of different assay methods and has been reported by several workers (Zheng and Wang, 2001; Dragland et al. 2003; Mimica-Dukic et al. 2003). Menthol derived from

M. piperita appears to affect cytosolic arylamine Nacetyltransferase (NAT) activity in the human liver tumor cell line J5 differentially dependent on dose (Lin et al. 2001) Ohara and Matsuhisa (2002) reported Peppermint for strong antitumor promoting activities against the non-12-O-tetradecanoylphorbol- 13-acetate (TPA)-type promoter, okadaic acid (OA), which promotes tumor formation by inhibiting protein phosphatase-2A. In addition to these *M. piperita* oil is also reported to possess anti-allergic activity, antiviral activity, fungicidal and antibacterial properties (McKay and Blumberg, 2006). Peppermint is taken internally as a tea, tincture, oil, or extract, and applied externally as a rub or liniment. Herbalists consider peppermint an astringent, antiseptic, antipruritic, antispasmodic, antiemetic, carminative, diaphoretic, mild bitter, analgesic, anticatarrhal, antimicrobial, rubefacient, stimulant, and emmenagogue (Hoffman and Lunder, 1984). Peppermint oil vapor is used as an inhalant for respiratory congestion. Peppermint tea is used to treat coughs, bronchitis, and inflammation of the oral mucosa and throat. It has traditionally been used to treat a variety of digestive complaints such as colic in infants, flatulence, diarrhea, indigestion, nausea and vomiting, morning sickness and anorexia, and as a spasmolytic to reduce gas and cramping. Peppermint is currently used to treat irritable bowel syndrome, Crohn's disease, ulcerative colitis, gall bladder and biliary tract disorders, and liver complaints (Blumenthal et al. 1998). Peppermint oil is used to relieve menstrual cramps. Peppermint oil is used externally for neuralgia, myalgia, headaches, migraines and chicken pox (Blumenthal et al. 1998; Bakkali et al. 2008).

2.3. Problem of drug resistance and need for novel anti-infective strategies

The emergence and spread of multi-drug resistance in pathogenic bacteria has become a significant global public health threat. Drug-resistant bacterial infections cause considerable patient mortality and morbidity, and rising antibiotic resistance is seriously threatening the vast medical advancements made possible by antibiotics over the past 70 years (Payne, 2008). Without developing innovative approaches to combat multidrug-resistant (MDR) pathogens, many fields of medicine will be severely affected, including surgery, premature infant care, cancer chemotherapy, care of the critically ill, and transplantation medicine; all of which are feasible only with the existence of effective antibiotic therapy (Worthington and Melander, 2013). Compounding the problem of rising bacterial resistance to currently approved

antibiotics is a lack of investment in antibiotic discovery by the pharmaceutical industry due to the inherently low rate of return for antibiotics compared to drugs targeted at chronic diseases (Spellberg et al. 2007). This situation is so dire that the World Health Organization has identified MDR bacteria as one of the top three threats to human health (Bassetti et al. 2011). Although the development of new antibiotics is one approach for the treatment of MDR bacterial infections, the fact remains that only two antibiotics have approved by U.S. FDA for human use from 2008 till 2012; neither of which are significantly active against Gram-negative bacteria (Spellberg, 2012) Furthermore, bacteria invariably develop resistance to any introduced drug that relies solely upon a bacteriostatic/bactericidal mechanism, and clinically significant resistance can appear in a period of just months to years following introduction of a new antibiotic into the clinic (Walsh, 2000). For example, daptomycin was introduced into the clinic in 2003, and less than a year later the emergence of resistance was observed in patients with *Enterococcus faecium* and MRSA infections (Dolgin, 2010). As a result, exploring alternative approaches to controlling bacterial infections are the need of the hour.

The problematic groups of common drug resistant bacteria include *Mycobacterim tuberculosis*, methicillin-resistant *Staphylococcus aureus* (MRSA), vancomycin resistant enterococci, ESBL producing MDR enteric bacteria, cystic fibrosis causing *Pseudomonas aeruginosa* (Naimi et al. 2003; Ahmad et al. 2009; WHO, 2012; Laxminarayan et al. 2012). The above problem is expected to increase in future due to the excessive and indiscriminate use of antibacterial drugs both in medical and non medical settings. The resistance in bacteria develops either through mutations or acquisition of new genes by genetic exchange mechanisms (transformation, transduction and conjugation) i.e. horizontal gene transfer. The major reservoir may be non-pathogenic strains, antibiotic producers itself (Martinez, 2008; D'Costa et al. 2006). Presence of a novel mechanism of genetic and cellular exchange by nanotubes formation between two related or unrelated bacteria indicating the presence more complex strategy adopted by bacteria for communication and gene exchange (Dubey and Yahuda, 2011).

Considering the current scenario of new antibacterial drug development by companies and increasing trends of multi-drug resistance (MDR) both in hospital,

community and environmental bacteria. There is an urgent need to develop strategies to combat problem of MDR. Various strategies have been described that includes (i) magic bullet concept i.e. discovery of novel antibacterial (ii) combinational approaches (iii) alternative strategies including antipathogenic strategy (Aqil et al. 2006; Ahmad et al. 2009). In the past decade antipathogenic drug targets have been identified and validated. It is expected that new drug molecules from nature and chemicals will be effective as it will not be the threat to the life of pathogens and will not select resistant pathogens due to drug selection pressure (Hentzer et al. 2003).

In the recent years target like quorum sensing which regulates many virulence factors expression in a cell density dependent manner have been identified as novel anti infective drug targets (Hentzer et al. 2003; Rasmussen and Givskov, 2006; Roman et al. 2013). Blocking communication of one's adversaries serves as an effective tactic to disrupt cooperative actions among individuals or groups. The knowledge gained over the last 40 years that bacteria commonly benefit from social interactions and intercellular signaling presents an opportunity to interfere with their ability to coordinate efforts to invade their hosts, whether human, animal, or plant. In fact, it is now realized that communication interference naturally exists in the microbial world, and it stands to reason that this ploy to gain an advantage over competitors was originally invented by bacteria (LaSarre and Federle, 2013).

Cell-to-cell communication in bacteria (quorum sensing [QS]) relies on small, secreted signaling molecules, much like hormones in higher organisms, to initiate coordinated responses across a population. In many cases, the responses elicited by QS signals are ones that contribute directly to pathogenesis through the synchronized production of virulence determinants, such as toxins, proteases, and other immune-evasive factors (Williams, 2007). Additionally, QS can contribute to behaviors that enable bacteria to resist antimicrobial compounds or drugs, such as biofilm development. If these efforts to coordinate were blocked, it is theorized that bacteria would lose their ability to mount an organized assault on the host's defense or immune system or would be less able to form organized community structures that promote pathogenesis, such as biofilms (Jayaraman and Wood, 2008). For some bacteria, working together as a group provides a means to build a defense or to surmount a barrier that individual bacterial cells find impossible to achieve. Blocking

interactions between bacteria would effectively force bacteria to live as individuals fending for themselves. One key advantage proposed in targeting quorum sensing is based on the premise that a treatment that does not suppress growth of a cell and will not exert a selective pressure to develop resistance to that treatment (Koh et al. 2012). Quorum sensing is not an essential process, and QS mutants in general have not displayed growth defects. Granted, interfering with the regulation of virulence factor production will likely reduce fitness for survival in certain situations, but if maintaining a delicate control over quorum-sensing-regulated genes is critical to the cell (and the exquisite layers of complexity found in many quorum-sensing pathways bolster this assumption), then developing resistance mechanisms against quorum-inhibiting therapies may be a difficult proposition for bacteria, which could help promote long-term efficacy of anti-QS therapies (LaSarre and Federle, 2013).

2.4. Quorum sensing

Quorum sensing (QS) is a bacterial cell–cell communication process that involves the production, detection, and response to extracellular signaling molecules called autoinducers (AIs). AIs accumulate in the environment as the bacterial population density increases, and bacteria monitor this information to track changes in their cell numbers and collectively alter gene expression (Novick et al. 1995; Seed et al. 1995; Novick and Geisinger 2008; Ng and Bassler 2009). QS gene products regulate a multitude of transcriptional programs in bacteria *in vitro* and *in vivo* (Engebrecht et al. 1983; Stevens et al. 1994; Zhu and Winans 1999, 2001; Federle and Bassler, 2003; Williams, 2007; Rutherford and Bassler, 2012). QS systems control biofilm formation (Asad and Opal, 2008), growth potential, sporulation, antibiotic resistance expression, DNA transfer, virulence expression, autolysis, oxidative stress tolerance, metabolic activity, motility, antibiotic synthesis by antibiotic-producing bacteria, sessile versus planktonic behavior, and most importantly genetic determinants of virulence (Novick and Geisinger 2008; Ng and Bassler 2009; Williams and Camara, 2009; Antunes et al. 2010; Rutherford and Bassler, 2012).

The discovery of QS is attributable to the pioneering work of three marine microbiologists – Nealson, Platt and Hastings (Nealson et al. 1970). An unusual form of symbiosis exists between the halophilic bacterium *Vibrio fischeri* and Hawaiian bobtail squid (*Euprymna scolopes*). The bioluminescent *V. fischeri* is taken up by

strategically placed light organs along the outer surface of the squid. When the bacterial population reaches a threshold concentration, the bacterium activates its luciferase operon to generate visible light. The bacteria benefit from its association with the squid, which provides a safe haven and a steady source of nutrients. The light source created by the bacterial enzymes provides the squid with an ingenious form of camouflage. The dark outline of the squid is silhouetted against the starlit sky on clear nights, rendering them readily visible from below by predatory fish (Fuqua and Greenberg, 2002). The light organs of the squid provide a starry sky camouflage thanks to the light source provided by the large aggregates of *V. fischeri*. Bioluminescence by this *Vibrio* species, and a closely related organism *Vibrio harveyi*, is activated only when large concentrations of bacteria are present. But how do individual bacterial cells sense when their population density is sufficient to generate bioluminescence? The answer came when *V. fischeri* was found to produce a soluble QS molecule that only induces transcription of the luciferase operon when neighboring bacterial populations are above a preset threshold concentration (Fuqua and Greenberg, 2002; Parsek and Greenberg, 2005; Williams, 2007).

Fuqua et al. (1994) introduced the term 'quorum sensing' to describe this phenomenon, and since the early 1990s there has been an exponential increase in the number of published papers presenting new data on the nature and function of quorum sensing systems in diverse bacterial genera. The term quorum sensing does not, however, adequately describe all situations where bacteria employ diffusible chemical signals. The size of the quorum, for example, is not fixed but will vary according to the relative rates of production and loss of signal molecule, i.e. it is dependent on the prevailing local environmental conditions. It is also possible for a single bacterial cell to switch from the 'non-quorate' to the 'quorate' state as has been observed for *Staphylococcus aureus* trapped within an endosome in endothelial cells (Qazi et al. 2001). In this context, 'diffusion sensing' or 'compartment sensing' are more appropriate terms since the signal molecule is supplying information with respect to the local environment rather than cell population density per se (Redfield 2002; Winzer et al. 2002). Quorum sensing might therefore be better considered as a special category of diffusion sensing where, in a given environment, the threshold concentration of signal molecule required to trigger a response can only be achieved by more than one cell (Redfield 2002; Winzer et al. 2002). Furthermore, it should be

remembered that quorum sensing, as the determinant of cell population density, is only one of many different environmental signals (e.g. temperature, pH, osmolarity, oxidative stress, nutrient deprivation) which bacterial cells must integrate in order to determine their optimal survival strategy (Withers et al. 2001). Thus, quorum sensing is an integral component of the global gene regulatory networks which are responsible for facilitating bacterial adaptation to environmental stress (Williams, 2007; Li and Tian, 2012).

2.5. Quorum sensing systems

In the recent years excellent review articles have been written by various workers on the QS systems (Miller and Bassler, 2001; Williams, 2007; Jayaraman and Wood, 2008; Atkinson and Williams, 2009; Antunes et al. 2010; Rutherford and Bassler, 2012; Kalia, 2013). A brief account has been given on the basis of recent literature survey. All quorum-sensing systems utilize small, secreted signaling molecules known as autoinducers (AIs). From an historical perspective, the most commonly studied autoinducers belong to one of the following three categories: acylated homoserine lactones (AHLs), used by Gram-negative bacteria (referred to as autoinducer-1 [AI-1]); peptide signals, used by Gram-positive bacteria; and autoinducer-2 (AI-2), used by both Gram-negative and Gram-positive bacteria. There are also other quorum sensing signals that go beyond these classes, including *Pseudomonas* quinolone signal (PQS), diffusible signal factor (DSF), and autoinducer-3 (AI-3), and new molecules will undoubtedly be discovered as the study of quorum sensing expands to species of bacteria yet to be investigated. QS system components and associated phenotypes of various bacterial species discussed in this review are outlined in Table R1

2.5.1. AHL-based quorum sensing

N-Acyl homoserine lactones (AHLs) are the molecules most commonly used by Gram-negative bacteria as quorum-sensing autoinducers. These molecules are comprised of an invariant homoserine lactone (HSL) ring attached to an acyl chain that can vary in length between 4 and 18 carbon atoms. In addition to acyl chain length, AHLs can also differ in the saturation state of the acyl chain and the oxidation state at position 3. Generally speaking, AHLs are biosynthesized by members of the LuxI family of AHL synthases using the substrates *S*-adenosylmethionine (SAM) and

an acylated acyl carrier protein (acyl-ACP) (Parsek et al. 1999). Exceptions to this are LuxM and AinS of *Vibrio harveyi* and *Vibrio fischeri*, respectively, which catalyze the same reaction but do not share homology with LuxI-type proteins (Gilson et al. 1995). In general, each AHL synthase predominantly makes a single type of AHL, although some synthases also produce additional AHLs in smaller amounts. Following synthesis, AHLs generally diffuse freely across the cell envelope and accumulate in the local environment. Alternatively, there is evidence that several AHLs may be actively transported across the cell membrane in certain bacterial strains (Evans et al. 1998; Pearson et al. 1999; Chan et al. 2007). Once a critical concentration threshold is achieved, interaction between the AHL and a LuxR-type receptor protein in the cytoplasm of the cell becomes favourable. LuxR family members are transcriptional regulators whose DNA-binding activities change upon ligand interaction, resulting in modulation of target gene regulation in response to AHL accumulation. A straightforward example of LuxI/R signaling is in *Chromobacterium violaceum* (Figure 1), which utilizes CviI, CviR, and the AHL C6HSL to regulate violacein production (McClean et al. 1997).

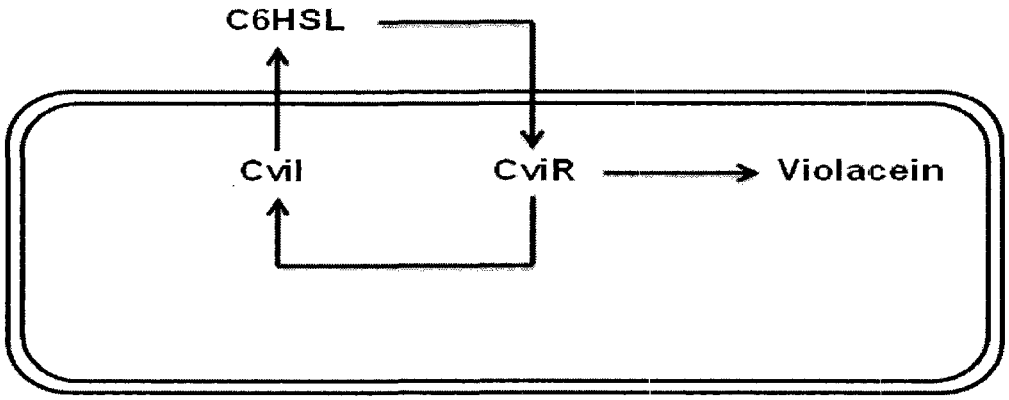


Figure R1. Simplified scheme of AHL based quorum sensing system in *C. violaceum*

Alternatively, some AHLs are detected by membrane- bound sensor kinases, such as LuxN of *V. harveyi*, that initiate phosphorelay signaling cascades following ligand binding (Bassler et al. 1993). In either case, each AHL receptor protein demonstrates some degree of AHL binding specificity based on the length, saturation, and oxidation of the AHL acyl chain. Accordingly, each bacterial species carries a cognate synthase/receptor pair that produces and responds to a specific AHL molecule. Exceptions to this are species that utilize multiple synthase/receptor pairs that generate and respond to distinct AHL molecules, as is the case for *P. aeruginosa*

(Jimenez et al. 2012), as well as species that carry so-called "solo" LuxR-type receptors that have no cognate LuxI-type synthase, such as QscR of *P. aeruginosa* and SidA of *Escherichia coli* (Subramoni and Venturi, 2009).

2.5.2. Peptide-based quorum sensing

Gram-positive bacteria do not harbor LuxI or LuxR homologues and instead utilize modified oligopeptides as auto inducer molecules. These are genetically encoded and are generated ribosomally within the cell. Specialized transporters are responsible for active transport of these peptides out of the cell due to their inability to permeate the cell membrane. At points between translation, export, and detection, peptides are subject to various modification events, including processing and/or cyclization. Detection of these peptide signals can occur either at the surface of the cell or intracellularly. Many peptide auto inducers known to date are detected by a membrane-bound sensor kinase, which switches its kinase/phosphatase activity in response to interaction with peptide, which alters the phosphorylation state of the cognate response regulator and ultimately results in activation or repression of QS target genes. Systems utilizing extracellular detection include the *agr* system of *Staphylococcus aureus* (Figure R2) and the *fsr* system of *Enterococcus faecalis*, which both control virulence factor production. The *agr* system of *S. aureus* centers around cyclic autoinducing peptides (AIPs) belonging to four distinct groups that interact with cognate AgrC sensor kinases of the same group to regulate exotoxin production and biofilm dispersal (Thoendel et al. 2011). The *fsr* system utilizes a different cyclic peptide, GBAP (gelatinase biosynthesis- activating pheromone), which is detected by the sensor kinase FsrC and induces production of gelatinase (Nakayama et al. 2001). Other bacteria utilize linear peptide autoinducers that are detected extracellularly, including the competence-inducing QS system of *Streptococcus pneumoniae* which is mediated by the competence- stimulating peptide (CSP) (Havarstein et al. 1995). Alternatively, some linear-peptide-based QS systems actively transport the autoinducers back into the cell where the peptide signal can interact directly with a cognate regulator to alter target gene expression. Such is the case for the PrgX system of *E. facaelis* and the PlcR and NprR systems of *Bacillus thuringiensis* (Rocha-Estrada et al. 2010, Dubois et al. 2012, Rocha et al. 2012).

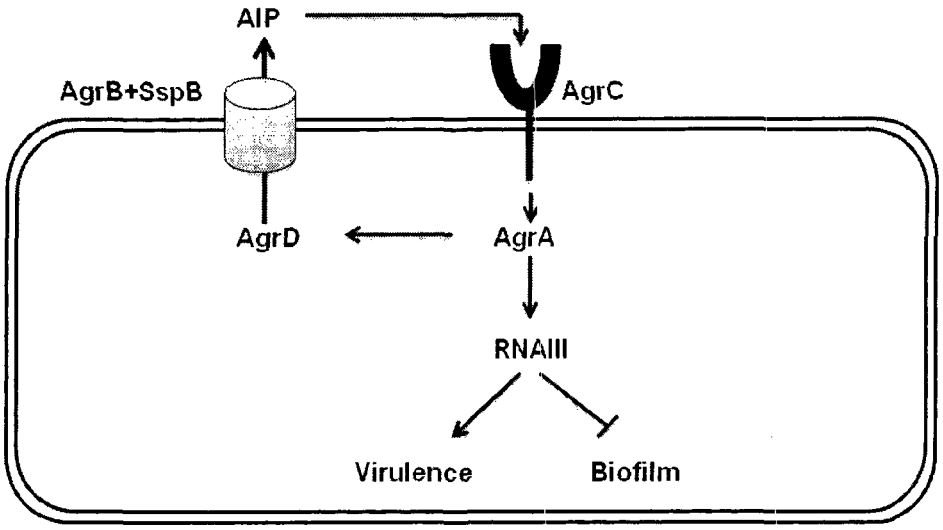


Figure R2. Peptide based quorum sensing system in *S. aureus*

2.5.3. AI-2-based quorum sensing

AI-2 is generated from the precursor *S*-adenosylhomocysteine (SAH) by the sequential enzymatic activities of 5 -methylthioadenosine/ *S*-adenosylhomocysteine nucleosidase (MTAN) (also known as Pfs) and the metalloenzyme LuxS (Schauder et al. 2001). The molecule resulting from these reactions is 4,5-dihydroxy-2,3-pentanedione (DPD), which is unstable in aqueous solution and undergoes spontaneous rearrangement into multiple inter-convertible cyclic furanone compounds that, as a group, are termed AI-2. AI-2 is thought to freely diffuse out of both Gram-negative and Gram-positive bacteria and, as is the case for all other QS signals, accumulates in the extracellular milieu. Many species produce and respond to AI-2, and AI-2 receptors have been identified in *V. harveyi* (Figure R3) and *Salmonella enterica* serovar Typhimurium. *V. harveyi* detects a boric acid-complexed form of AI-2 via the LuxP/LuxQ receptor/sensor kinase complex (Chen et al. 2002; Neiditch et al. 2006). In contrast, the *S. Typhimurium* transporter LsrB interacts with a non-borated form of AI-2, leading to its internalization, phosphorylation, and interaction with the cytoplasmic transcriptional regulator LsrR (Taga et al. 2003). Thus, different bacterial species can detect different forms of AI-2, and detection can take place either extra- or intracellularly, depending on the bacterium.

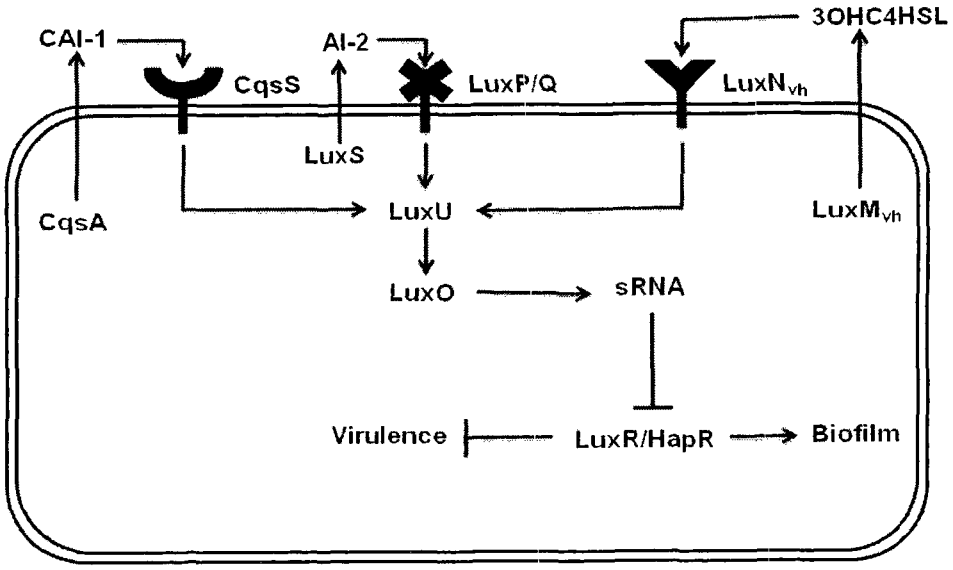


Figure R3. AI-2 based quorum sensing system in *V. harveyi*

2.5.4. Quorum-sensing systems utilizing other autoinducers

As mentioned earlier, in addition to the three main categories of autoinducers, there are also other autoinducer molecules that do not fit within these classes. 2-Heptyl-3-hydroxy-4(1*H*)-quinolone (*Pseudomonas* quinolone signal [PQS]) and its precursor 2-hep- tyl-4(1*H*)-hydroxyquinoline (HHQ) are the biosynthetic products of the *pqsABCDE* operon, which mediates the condensation of anthranilate and a β-keto-fatty acid. Both PQS and HHQ act as QS autoinducers via their interaction with the transcriptional regulator PqsR, which results in modulation of target gene expression. The only structural difference between the two molecules, an additional hydroxyl group added to the 3 carbon atom of HHQ by the enzyme PqsH, appears to be important for the ability of PQS to function in iron acquisition in addition to its role in quorum sensing. Although only *P. aeruginosa* produces PQS, other species of *Pseudomonas* as well as *Burkholderia* spp. utilize HHQ as a quorum-sensing signal (Dubern and Diggle, 2007; Jimenez et al. 2012). The signaling molecule *cis*-11-methyl-2-dodecenoic acid (diffusible signal factor [DSF]) was originally identified in *Xanthomonas campestris* pv. campestris but has since been determined to belong to a family of QS signals utilized by a variety of bacterial species, including *Burkholderia cenocepacia* and *Xylella fastidiosa*. Production of DSF or *Burkholderia cenocepacia* DSF (BDSF) requires RpfB of *Xanthomonas campestris* or its homologue Bcam0581 of *B. cenocepacia*, respectively (Deng et al. 2011; Bi et al. 2012), and it was recently shown that BDSF is generated from the acyl carrier protein (ACP) thioester of 3-

hydroxydecanoic acid by sequential Bcam0581-catalyzed dehydration and thioester cleavage reactions (Bi et al. 2012). DSF family signal detection appears to differ between species, although both currently known mechanisms share the common feature of altering intracellular levels of cyclic di-GMP (c-di-GMP) (Deng et al. 2011; Deng et al. 2012).

Upon accumulation of DSF in the extracellular milieu of *Xanthomonas campestris*, it interacts with the sensor kinase RpfC to induce a phosphorelay cascade that activates the response regulator RpfG. Activated RpfG functions to degrade c-di-GMP, and the subsequent decrease in the intracellular concentration of c-di-GMP results in activation of Clp regulators, which directly and/or indirectly regulate target gene expression. DSF signaling in *Xylella fastidiosa* appears to require the same genetic components; however, the precise system architecture appears to differ from that of *Xanthomonas campestris* and needs further characterization (Deng et al. 2011).

In *B. cenocepacia*, BDSF is detected by the receptor protein RpfR, which contains a PAS domain, a GGDEF domain, and an EAL domain. The phosphodiesterase activity of RpfR conferred by the EAL domain is stimulated upon interaction of RpfR with BDSF, thereby directly linking BDSF signal perception with reduction in c-di-GMP levels (Deng et al. 2012). The autoinducer AI-3 is an aromatic signaling molecule produced by bacteria of the human intestinal microflora as well as certain enteric pathogens. The molecular structure of AI-3 is unknown, as is the gene responsible for AI-3 production. AI-3 detection by the enteric pathogens *Escherichia coli* and *Salmonella Typhimurium* occurs via the sensor kinase QseC, which then phosphorylates the response regulator QseB to activate transcription of target genes. The same QseC/B two-component system is also used for the bacterial detection of epinephrine and norepinephrine produced by the host, and thus it is hypothesized that the structure of AI-3 may resemble that of the two hormones (Kendall and Sperandio 2007, Moreira et al. 2010).

Other QS systems found in various *Vibrio* sp. as well as in *Legionella pneumophila* utilize _-hydroxyketones (AHKs) as signaling molecules. The *Vibrio cholerae* and *V. harveyi* genomes contain the *cqs* gene cluster, which is responsible for the production and There are various reports indicating that *V. cholerae* and *V. harveyi* simultaneously integrate CAI-1, AHL, and AI-2 signaling to regulate

virulence-related processes via use of common downstream regulatory proteins (Tiaden et al. 2010; Rutherford and Bassler, 2012; LeSarre and Federle, 2013).

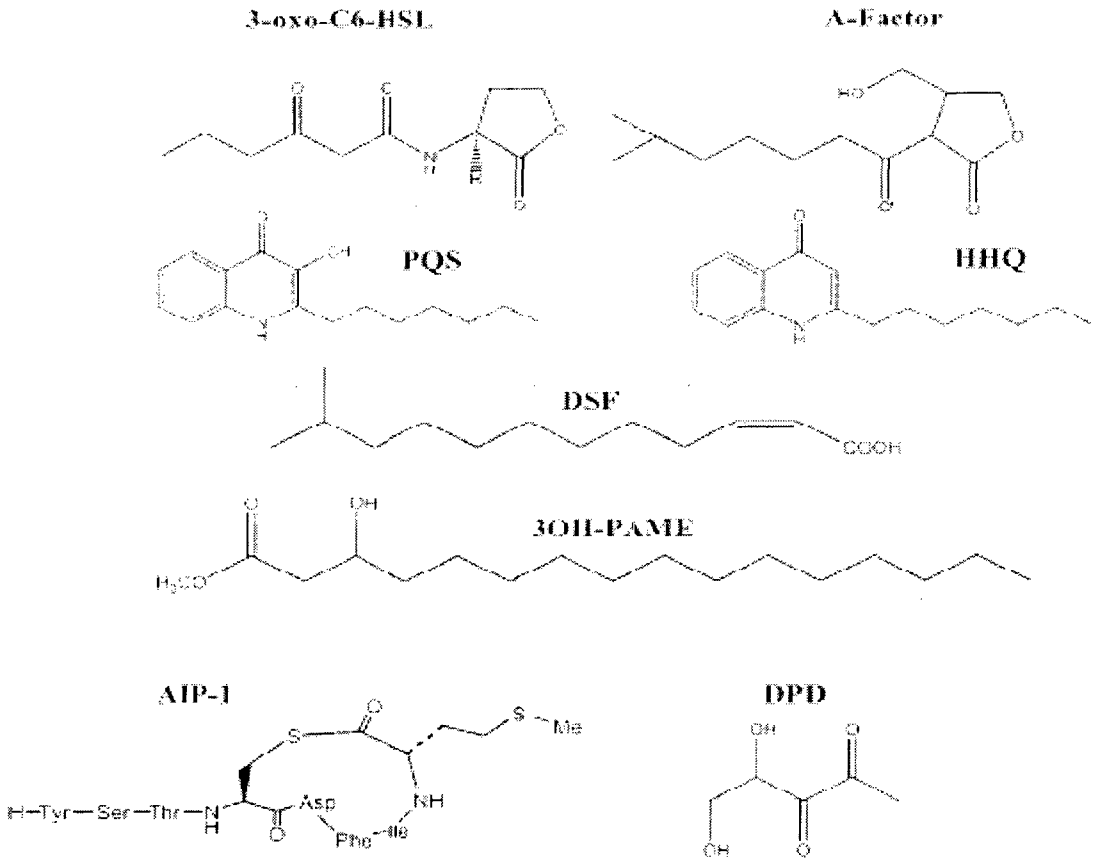


Figure R4. Examples of the structures of some representative QS signal molecules. 3-oxo- C6-HSL, N-(3-oxohexanoyl)-L-homoserine lactone; A-Factor, 2-isocapryloyl-3-hydroxymethyl- c-butyrolactone; PQS, pseudomonas quinolone signal, 2-heptyl-3-hydroxy-4(1H)- quinolone; HHQ, 2-heptyl-4(1H)-quinolone; DSF, 'diffusible factor', cis-11-methyl-2-dodecenoic acid; 3OH-PAME, hydroxyl-palmitic acid methyl ester; AIP-1, staphylococcal autoinducing peptide 1; DPD, the AI-2 precursor, 4,5 dihydroxy-2,3-pentanedione

Table R1: Quorum-sensing systems utilized by selected bacteria and their associated phenotypes

Organism	Signal	Synthase(s)	Receptor(s)	Selected phenotypes	References
Pseudomonas aeruginosa	C4HSL	RhlI	RhlR	Exoenzymes, virulence,	Dubern and
	3OC12HSL	LasI	LasR, QscR	biofilm formation,	Diggle, 2008;
	HHQ, PQS	PqsA to –D, PqsH	PqsR	motility, ironacquisition, pyocyanin	Jimenez et al. 2012
Pseudomonas syringae	3OC6HSL	AhlI	AhlR	EPS, plant colonization	Von Bodman et al. 2003
Erwinia caratovora	3OC6HSL	CarI	CarR, ExpR, VirR	Carbapenem, exoenzymes, virulence	Barnard and Salmond, 2007
Pantoea stewartii	3OC6-HSL	EsaI	EsaR	Adhesion, EPS, plant colonization	Von Bodman et al. 2003
Agrobacterium tumefaciens	3OC8HSL	TraI	TraR	Ti plasmid conjugation, virulence	Von Bodman et al. 2003
Chromobacterium violaceum	C6HSL	CviI	CviR	Exoenzymes, antibiotics, violacein	McClean et al. 1997
Serratia liquefaciens	C4HSL	SwrI	SwrR	Swarming motility, biofilm formation	Labbate et al. 2004
Vibrio harveyi	3OHC4HSL	LuxM	LuxN	Bioluminescence,	Milton, 2006; Ng
	AI-2	LuxS	LuxP	siderophores, protease	and Bassler, 2009
	CAI-1	CqsA	CqsS	and EPS production, virulence	
Vibrio cholera	AI-2	LuxS	LuxP	Virulence, biofilm	Milton, 2006; Ng
	CAI-1	CqsA	CqsS	formation, EPS	and Bassler, 2009
Vibrio fischeri	3OC6HSL	LuxI	LuxR	Bioluminescence, host	Milton, 2006
	C8HSL	AinS	AinR	colonization, motility	
	AI-2	LuxS	LuxP		

	3OC8HSL[a]	NA[a]	SdiA	Motility, acid resistance	Soares and Ahmer, 2011
Escherichia coli/Salmonella typhimurium	AI-2	Luxs	LsrB	Lsr operon expression (AI-2 uptake)	Kendall and Sperandio, 2007
	AI-3	Unknown	QseC	Virulence, motility, biofilm formation	Moreira et al. 2010
Staphylococcus aureus	AIP	agrD[b]	AgrC	Virulence, exotoxins, biofilm depersal	Thoendel et al. 2011
Enterococcus faecalis	GBAP	fsrD[b]	FsrC	Gelatinase, protease	Nakayama et al. 2001
	cCF10	ccfA[b]	PrgX	Adhesion, conjugation	Rocha-Estrada et al. 2011
Streptococcus pneumoniae	CSP	comC[b]	ComD	Competence, virulence, autolysis	Guiral et al. 2005; Kowalko and Sebert, 2008
Bacillus thuringiensis	PapR	papR[b]	PlcR	Exoenzymes	Rocha-Estrada et al. 2011
	NprX	nprRB[b,c]	NprR	Toxins, spotulation, necrotrophism	Dubois et al. 2012; Rocha et al. 2012
Xanthomonas campestris	DSF	RpfB, RpfF	RpfC	Virulence, biofilm dispersal, EPS	Deng et al. 2011
Aeromonas hydrophila	C4HSL	AhyR	AhyI	Biofilm, exoproteases	Swift et al. 1997
Burkholderia cenocepacia	C6-HSL, C8-HSL	CepR, CciR	CepI, CciI	Exoenzymes, biofilm formation, swarming motility, siderophore, virulence	Sokol et al. 2003

2.6. Quorum sensing in the model pathogen *Pseudomonas aeruginosa*

Pseudomonas aeruginosa is a Gram-negative bacterium capable of surviving in a wide range of environments. This organism is an opportunistic pathogen and it is commonly associated with nosocomial infections and infections of severely burned individuals, and is a leading cause of death in severe respiratory infections, such as chronic lung infections in cystic fibrosis patients (Bodey et al., 1983; Koch and Hoiby, 1993; Bendiak and Ratjen, 2009). Infections with *P. aeruginosa* are difficult to eradicate, due to their high levels of antibiotic resistance and growth in biofilms (Driscoll et al. 2007).This human pathogen has been used extensively for studies on quorum-sensing inhibition for multiple reasons. First, the quorum-sensing network of *P. aeruginosa* is one of the best characterized quorum-sensing systems to date. Second, quorum sensing regulates the expression of numerous virulence-related products and has been shown to be important for *P. aeruginosa* pathogenesis in various model infection systems. Third, this pathogen poses a large burden on the medical community due to its extensive resistance to antibiotics and the current lack of effective treatment options (Jimenez et al. 2012), and thus discovery of quorum quenching inhibitors amenable to therapeutic applications would be of high value.

Quorum sensing in *P. aeruginosa* is a complex network comprised of at least three distinct signal/receptor pairs that function in a hierarchical fashion (Figure R5). *P. aeruginosa* has two LuxIR homologue pairs, LasIR and RhlIR, which produce and detect the AHL signaling molecules 3OC12HSL and C4HSL, respectively. The LasR-3OC12HSL complex induces expression of LasI as well as expression of RhlR, thereby positively regulating both AHL-based QS systems. The RhlR-C4HSL complex subsequently mediates its own autoinduction but has no direct effect on the LasR system. LasR and RhlR additionally positively and negatively regulate, respectively, expression of genes involved in the third quorum-sensing loop, which is based on the autoinducer *Pseudomonas* quinolone signal (PQS). PQS autoinduces its own production while concomitantly enhancing RhlR expression, thereby self-limiting its expression via an extended negative-feedback loop. PQS has no direct regulatory activity on the Las QS system. Each QS system also regulates various virulence factor-encoding genes, some of which are coregulated by the other QS systems. Additionally, there is also a solo LurR-type protein, QscR that binds 3-O-

C12HSL and subsequently inhibits both the *las* and *rhl* QS systems by multiple mechanisms (Dubern and Diggle, 2008; Jimenez et al. 2012).

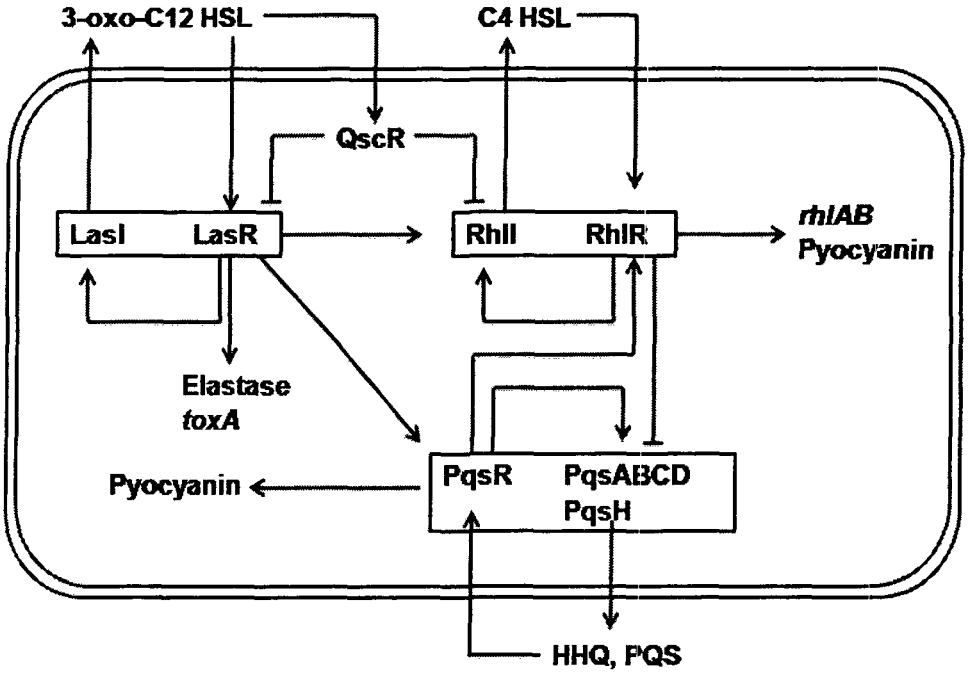


Figure R5. Diagramatic representation of *P. aeruginosa* quorum-sensing networks.

Multiple virulence factors are involved in the pathogenesis and disease. Development of *P. aeruginosa* virulence factors includes secreted factors (such as proteases) and cell-associated factors (such as lipopolysaccharide and flagella), as well as the ability to form biofilms (Lyczak et al. 2000). Quorum sensing regulates the production of several extracellular virulence factors, promotes biofilm maturation and regulates the expression of antibiotic efflux pumps, meaning that it has a key role in the pathogenesis of *P. aeruginosa* as depicted in figure R6 (Fuqua et al. 2001; Swift et al. 2001; Whitehead et al. 2001; Wagner et al. 2003; Diggle et al. 2006; Schuster and Greenberg, 2006; Dekimpe and Deziel, 2009). The las and rhl systems regulate the timing and production of multiple virulence factors, including elastase, alkaline protease, exotoxin A, rhamnolipids, pyocyanin, lectins and superoxidase dismutase (Schuster et al. 2003; Smith and Iglewski, 2003). The expression of these two quorum sensing systems has also been linked to the regulation of biofilm formation.

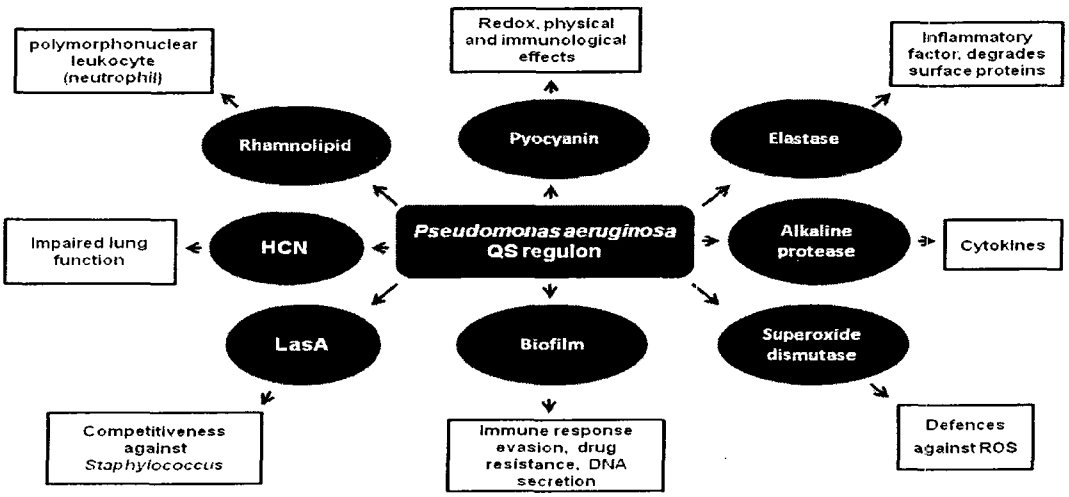


Figure R6: QS regulated virulence traits in *Pseudomonas aeruginosa*

Quorum sensing signalling may start in the early stages of biofilm development, which is characterized by microcolony formation, where lasI mutants are unable to form structurally normal biofilms (Davies et al. 1998). Expression of the *lasI* gene is maximal at day 4 of Biofilm development, decreasing between days 6 and 8. The expression of rhlI fluctuates during biofilm development and phenotypes of biofilm development with a rhlI mutant vary, according to the media and model used, supposedly due to different iron levels present (Davies et al. 1998; Yoon et al. 2002; Patriquin et al. 2008).

Significant concentrations of the AI molecules 3-OC12-HSL and C4-HSL have been detected in sputum of CF patients colonized with *P. aeruginosa* (Singh et al. 2000; Erickson et al. 2002). This may indicate that quorum sensing is active during *P. aeruginosa* colonization of CF patients; however, it is important to note that quorum-sensing deficient *P. aeruginosa* strains are often isolated from CF patients (Erickson et al. 2002; Schaber et al. 2004; Le Berre et al. 2008; Karatuna and Yagci, 2010).

The third *P. aeruginosa* AI molecule (*Pseudomonas* quinolone signal; PQS) belongs to the 2-alkyl-4-quinolone (AQ) family and it is synthesized via the *pqsABCD* genes in the *pqsABCDE* operon, which are responsible for the synthesis of 2-heptyl-4-quinolone (HHQ), the immediate PQS precursor (Diggle et al. 2006). Through transcriptome analysis, over 90 genes were found to be regulated by the *pqs* system (Deziel et al. 2005; Bredenbruch et al. 2006). PQS has been shown to affect biofilm formation and to regulate several virulence factors in *P. aeruginosa*, including

elastase, pyocyanin and LecA lectin, and it is considered essential for full virulence in multiple hosts (Rahme et al. 1997, 2000; Cao et al. 2001). PQS has been found in sputum, bronchoalveolar fluid and mucopurulent fluid from CF patients, suggesting that it may play an important role during the infection process (Collier et al. 2002). Besides controlling bacterial virulence, PQS and HHQ have been shown to downregulate the host immune response through NFkB (Kim et al., 2009).

Because of the significant role of quorum sensing in the regulation of an array of virulence factors in *P. aeruginosa*, significant efforts have been made to discover molecules that inhibit quorum sensing in this organism.

2.7. Quorum sensing interference strategies

In the last decade concept of antipathogenic drug has gained importance in possible treatment of bacterial diseases by the attenuation/blocking of quorum sensing in bacteria (Hentzer and Givskov, 2003). QS systems generally offer three major points of attack for the development of QS inhibitors: the signal generator (LuxI homologue), the signal molecule (AHL) and the signal receptor (LuxR homologue) (Rasmussen and Givskov, 2006). The major strategies of blocking the quorum sensing are summarized in figure R7.

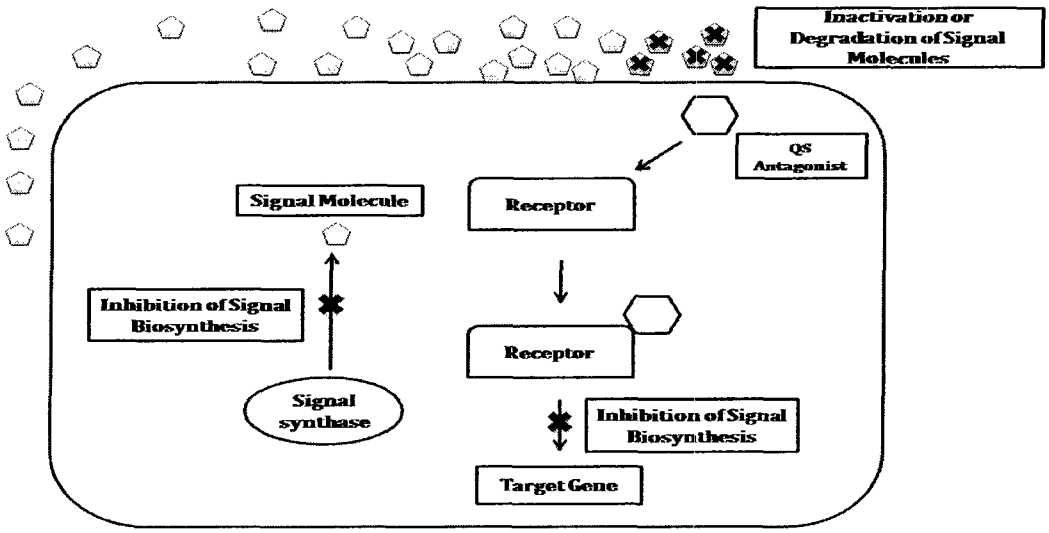


Figure R7. Schematic view of different strategies of QS inhibition

2.7.1. Interfering with the signal generation: AHL molecules are synthesized by AHL synthase/I protein using corresponding acyl chain derived from fatty acid biosynthesis pathway and S-adenosylmethionine (SAM) (Schaefer et al. 1996). Though small chain AHLs are freely diffusible, long chain AHLs require the use of

efflux pumps like MexABOprM for their transit across the bacterial membrane (Pearson et al. 1999). Any compound inhibiting the fatty acid biosynthesis, SAM biosynthesis, I protein synthesis or efflux pumps would work at the level of basal QS signal generation and could function as a QSI. Substrate analogues like butyryl-S-adenosylmethionine have been found to block the AHL production *in vitro* but these homologues are likely to affect the central pathways of amino acid although *in vivo* studies have not been conducted. Hence this is not a very popular strategy for the QSI development (Rasmussen and Givskov, 2006).

2.7.2. Degradation of the signal molecule: Inactivation or complete degradation of the AHL signal molecules can be achieved by either of these methods: chemical degradation, enzymatic destruction or metabolism of the AHL molecules. AHL-lactonase, AHL-acylase and paraoxonase enzymes play a role in the degradation of AHL and this enzymatic disruption of QS is typically termed quorum quenching (QQ). Metabolising the AHL molecules is also one of the strategies adopted by certain bacteria so that they are no longer available for QS-related processes. *P. aeruginosa* PAI-A and *Variovorax paradoxus* use AHLs as sole source of energy, carbon and nitrogen (Huang et al. 2003; Bhardwaj et al. 2013).

2.7.3. Signal inhibition/interfering with signal reception: This involves the non-enzymatic inhibition of AHL molecules. Analogues of AHL signal molecules have been designed to block the receptor. These analogues have been designed either by modifications in the acyl side chain or in the lactone ring or in both these moieties of the AHL molecule. Similar to the natural AHL antagonists, several molecules have been designed by modifying the structure of AHLs (Ni et al. 2009) or by modification of the natural halogenated furanones that have been shown to be effective in *P. aeruginosa* lung infection in mouse (Wu et al. 2004) or *Vibrio anguillarum* infection in trout (Rasch et al. 2004).

Similar to the inhibition of AI-1 pathways, AI-2 and AI-3 could also be targeted. AI-2 QS system offers a target for design of broad-spectrum QSIs. DPD is synthesized from S-adenosyl-L-homocysteine (SAH) in a two step enzymatic process which involves methylthioadenosine (MTA) nucleosidase (Pfs) and S-ribosylhomocysteinase (LuxS). The enzyme inhibitors can inhibit AI-2 and also AI-1 as the signals from both these systems are derived from SAM. Alternatively,

interfering with signal reception or signal transduction process could also inhibit AI-2 pathway. QseC, the receptor molecule for AI-3 is an attractive drug target as it is not found in mammals. Being present in many important animal and plant pathogens, compounds blocking this histidine sensor kinase/receptor have been pursued as broad spectrum drugs (Rasko et al. 2008; Kalia, 2013).

2.8. Criteria for selecting quorum sensing inhibitors

For selecting an effective QSI, it has been proposed that it should meet at least the following few criteria as described by Hentzer and Givskov (2003); Rasmussen and Givskov (2006) and Vattem et al. (2007). An effective QS inhibitor should be (a) a small molecule with ability to efficiently reduce QS regulated gene expression, (b) highly specific for a given QS regulator with no adverse effect(s) on the bacteria or the host, (c) chemically stable and resistant to degradation by various host metabolic systems, and (d) preferably longer than the native AHL, (e) QSIs should not be toxic to the eukaryotic hosts where the bacterial infections would be treated or to the bacteria being targeted, (f) They should not interfere with the basal metabolic processes of a bacterial cell like protein synthesis, DNA metabolism, cell wall formation that are targets for the development of drug resistance. As a consequence of these characteristics of a QSI, the bacteria are not likely to become resistant to such drug(s) and these compounds are not likely to affect the population of beneficial bacteria present in the communities harbouring the host (Rasmussen et al. 2005a). Finally, QSIs, which do not show antigenicity due to their low molecular weights, are expected to expedite drug discovery against infectious diseases.

2.9. Bacterial reporters to detect QS signals and screening quorum sensing inhibitors

One of the most important prerequisites for circumventing the pathogenicity issues related to QS is its detection. The need for biosensors was realized quite early primarily because of the increasing variation in the QS signals produced by diverse organisms. In order to establish the link between cause and effects, AHL and AI-2 reporters were developed over a period of time by different researchers listed in Table R2 (Bassler et al. 1993; Fuqua and Winans, 1996; Givskov et al. 1996; McClean et al. 1997; Shaw et al. 1997; Swift et al. 1997; Wood et al. 1997; Lindum et al. 1998; Winson et al. 1998; Steidle et al. 2001; Venturi, 2006). These biomonitor strains

allow sensitive, quantitative and real time detection of QS signals such as AHLs. In most of the biomonitor strains known so far, the QS regulated promoter is fused to the lux operon or lacZ. Although, these reporter strains have a functional regulator protein, they lack the AHL synthase enzyme. The promoter activity gets induced by exogenous QS signals. Here, the receptor gets activated by the presence of AHLs, which binds to its cognate LuxI promoter and initiates the expression of certain genes. The expression of the relevant genes is proportional to the concentration of the signal molecules (Swift et al. 1997; Winson et al. 1998). In brief, it mimics the natural QS system with certain easily identifiable phenotypes (Table R2). Although each reporter strain detects a set of QS signals, their complementarities allow detection of a wide range of AHLs and even AHL analogues or mimics (Teplitski et al. 2000; Steidle et al. 2001; Someya et al. 2009).

Chromobacterium violaceum has high sensitivity for QS signal compounds with 4–6 carbon acyl side chains, *E. coli* harbouring pSB410 is effective for 6–8 carbon side chains and pSB1075 is sensitive for detecting AHLs with 10–14 carbon side chains lengths (McClean et al. 1997; Winson et al. 1998). The inability of *C. violaceum* CV026 biosensor to detect 3-hydroxy derivatives of AHL can prove helpful in elucidating potential cases where *P. fluorescens* may be present (Cha et al. 1998). Another equally effective biosensor for long chain AHL inhibitor screening is *Agrobacterium tumefaciens* NT1 (traR, tra::lacZ749). It contains a lacZ fusion in the tral gene of pTiC58, which is induced to produce the enzyme β-galactosidase. The degradation of 5-bromo-4-chloro-3-indoyl β-D-galactopyranoside (X-gal) results in the appearance of blue colour. The best part of this biosensor strain is its ability to respond to a wide range of AHLs at very low concentrations (Shaw et al. 1997).

The third class of reporter strain needed for identifying QSIs which may target long-chain AHLs C16-C20 is represented by *S. meliloti* Rm41 sinI::lacZ (pJNSinR) (Llamas et al. 2004) (Table R2). A more recent addition to reporters for detecting long-chain AHLs is *C. violaceum* VIR24, which was derived from *C. violaceum* type strain ATCC12472 (Someya et al. 2009). *In vitro* methods including high throughput genetic tools have proven effective in screening of non-toxic QSIs from natural sources and for elucidating their effects Rasmussen et al. 2005a, b; Bjarnsholt et al. 2010).

Table R2: Bacterial reporter strains used to detect quorum sensing signals.

Organism	Quorum sensing signal detected	Phenotype	References
Agrobacterium tumefaciens A136 [traI-lacZ fusion (pCF218)(pCF372)]	C6HSL to C14-HSL	β-galactosidase activity	Fuqua and Winans, 1996
A. tumefaciens strain NT1 (pDCI41E33 containing a traG::lacZ fusion)	AHLs with 3-oxo-, 3-hydroxy-, and 3-unsubstituted side chains of all lengths, (C6-C14HSL) with the exception of C4HSL	β-galactosidase activity	Shaw et al. 1997
Chromobacterium violaceum strain CV026–CviR receptor	Wide host range of AHLs	Violacein pigment production	McClean et al. 1997
Escherichia coli plasmid carrying a luxCDABE cassette activated by AhyRI' receptor of *Aeromonas hydrophila* (pSB536)	C4HSL	Bioluminescent	Swift et al. 1997
E. coli plasmid carrying a luxCDABE cassette activated by AhyR receptor of *A. hydrophila* (pSB403)	Wide host range of AHLs	Bioluminescent	Winson et al. 1998
E. coli JM109 plasmid carrying a luxCDABE cassette activated by LuxR receptor of *Vibrio fischeri* (pSB401)	C6HSL	Bioluminescent	Winson et al.1998
E. coli JM109 plasmid carrying a luxCDABE cassette activated by LasR receptor of *Pseudomonas aeruginosa* (pSB1075)	C12HSL	Bioluminescent	Winson et al.1998
E. coli JM109 plasmid carrying a luxCDABE cassette activated by RhlR receptor of *P. aeruginosa*	C4HSL	Bioluminescent	Winson et al. 1998
Pseudomonas aureofaciens strain 30-84I	C6HSL	Phenazine antibiotic production	Wood et al. 1997
Pseudomonas putida 117(pAS-C8)-CepR receptor	C8HSL	Green Fluorescent	Steidle et al. 2001

P. putida IsoF/gfp	3OC12HSL	Protein Fluorescence	Venturi, 2006
Serratia liquefaciens strain MG44	C4HSL	Biosurfactant production	Lindum et al.1998
S. liquefaciens strain PL10 — LuxAB reporter	C4HSL	Bioluminescent	Lindum et al., 1998
Sinorhizobium meliloti Rm41 sinI::lacZ (pJNSinR)	C16–C20HSL	β-galactosidase activity	Llamas et al., 2004
Vibrio harveyi BB170 — LuxP receptor	AI-2	Bioluminescent	Bassler et al., 1993
V. harveyi BB886 — LuxP receptor	AI-1	Bioluminescent	Bassler et al., 1993

2.10. Quorum sensing inhibitors

QS plays an important role in the virulence factor production of several pathogenic bacteria. Consequently, compounds that interfere with the QS system to attenuate virulence and pathogenicity are termed as anti-QS compounds. Therefore, the use of anti-QS compounds may be of great interest in preventing/treating bacterial infections as these compounds neither kill the bacteria nor stop its growth and are less expected to develop resistance (Kalia, 2013). Search for effective, safe and stable anti-QS compounds has now been considered from various natural sources and also from synthetic compounds. Recent developments on QSIs has been reviewed and described here.

2.10.1. Furanones and other inhibitors

Anti-QS agents were first characterized in the red marine alga, *Delisea pulchura*. This alga was investigated for its anti-fouling properties, and was found to contain halogenated furanones, compounds which block AHLs via competitive inhibition and destabilization of LuxR. The structural similarity allows furanones to competitively inhibit the action of AHL signaling molecules (Manefield et al. 2002). A well studied furanone isolated from *D. pulchra* has been shown to have direct effect on biofilm formation is (5Z)-4-bromo-5- (bromomethylene)-3-butyl-2-(5H)-furanone (Figure R7). Initially, Ren et al. (2001) have shown that the furanone inhibited swarming and biofilm formation of *E.coli* XL1-blue on mild steel surfaces with little toxicity to the bacteria. AHL as well as AI-2 based QS systems are inhibited by furanones as these compounds are structural mimics of QS signals (lactones and tetrahydrofuran rings) in both these QS systems (Ren et al. 2004). Though initially it was believed that

furanones competitively inhibit the binding of autoinducers to their receptors, but it is now evident that halogenated furanones destabilize and accelerate the turnover of LuxR in *V. fischeri* and *V. harveyi*. This impairs the ability of LuxR to bind DNA and initiate transcription (Lowery et al. 2008).

The improved furanone compound, furanone 4 can increase the susceptibility of *P. aeruginosa* biofilm to tobramycin (Pan and Ren, 2009). Brominated furanones have also been found to be effective against Gram-positive bacteria as well as fungi (Holmstrom and Kjellberg, 2001; Pan and Ren, 2009). While natural furanones have shown considerable QSI activity in some species, a number of synthetic analogues have been shown to inhibit biofilm formation. One such compound is called Furanone 56 (Figure R8), was first demonstrated by Hentzer et al. (2002) to inhibit quorum sensing in *P. aeruginosa*. Furanone 56, unlike many other active furanones lacks both a side chain and bromine on the furanone ring. It was shown to have little effect on bacterial growth, protein synthesis and early biofilm formation. However, it was demonstrated that it could penetrate the biofilm matrix and interfere with biofilm maturation, presumable by disrupting QS gene expression. The effectiveness of Furanone 56, as well as that of a related furanone, for the treatment of lung infections in mice models has also been demonstrated (Wu et al. 2004). Both compounds have been shown to disrupt AHL quorum sensing, ultimately resulting in accelerated lung clearance and prolonged survival time of the mice. Since most of these furanones contain halogens, making them unsuitable for human use. The furanones investigated are too reactive and may be too toxic for treatment of bacterial infections in human (Hentzer and Givskov, 2003; Kociolek, 2009).

In addition to the furanones isolated from the marine algae several other inhibitors of marine origin have also been reported for their anti-QS properties. Korean red alga-*Ahnfeltiopsis flabelliformis* produces α-D-galactopyranosyl-(1→2)-glycerol (Floridoside), betonicine and isoethionic acid as anti-QS molecules (Figure R8) (Kim et al. 2007). Compounds have been reported from cyanobacteria with abilities to inhibit QS gene expressions. Malyngolide and lyngbyoic acid (Figure R8) isolated from *Lyngbya majuscula* were able to inhibit violacein production in *C. violaceum*, and elastase and pyocyanin production in *P. aeruginosa*, respectively (Dobretsov et al. 2010; Kwan et al. 2011). Two other compounds malyngamide C and

8-epi-malygamide isolated from the above alga were able to inhibit QS activities in *P. aeruginosa* (Kwan et al. 2010). Anti-quorum sensing activity has also been demonstrated by *Asparagopsis taxiformis* (marine macro alga) in *C. violaceum* CVO26 and *Serratia liquefaciens* MG44 biosensor strains (Jha et al. 2013). Bacterial strains *Stenotrophomonas maltophila* and *Delftia tsuruhatensis* isolated from the coral *Acropora digitifera* have also been reported to inhibit the expression of QS controlled virulence traits in *P. aeruginosa* (Bakkiaraj et al. 2012).

Fungi are known to produce secondary metabolites like antibiotics. Around 33 *Penicillium* spp. have been found to produce QS inhibitory compounds-patulin and penicillic acid (Figure R8) (Rasmussen et al. 2005b). Using mouse pulmonary infection model, the use of patulin could significantly reduce the infections caused by *P. aeruginosa* in mice although not suitable for human use to toxicity. Natural pigments produced by *Auricularia auricular* with ability to act as QSIs were observed to inhibit violacein production in *C. violaceum* (Zhu et al. 2011).

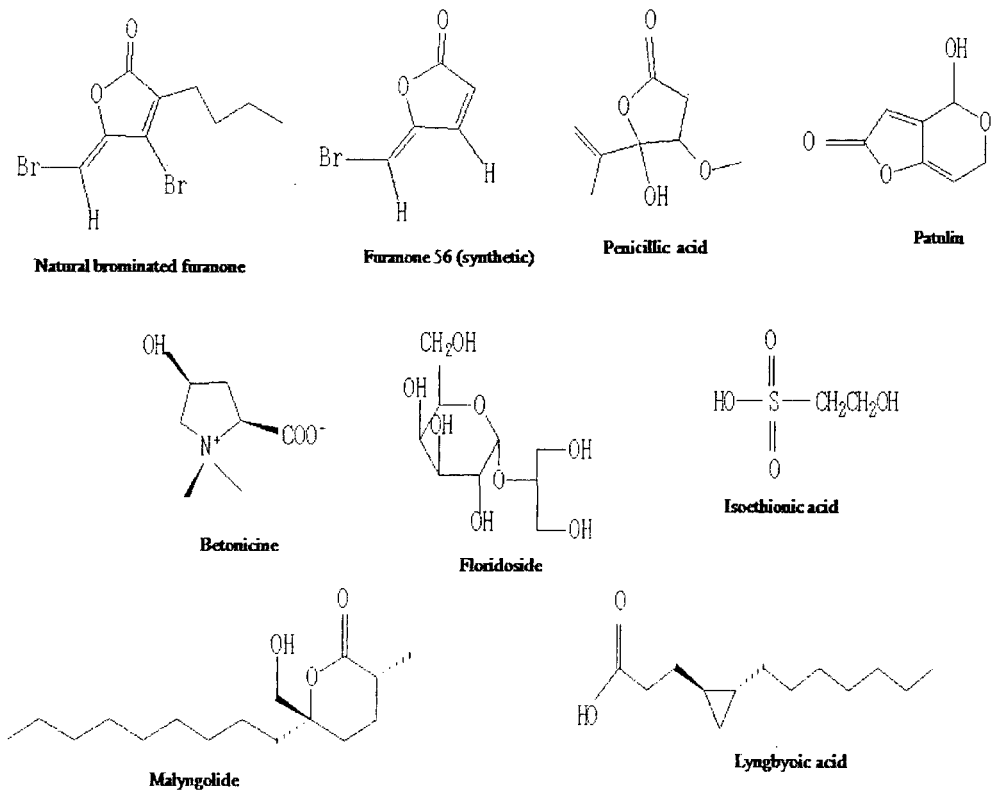


Figure R8. Inhbitors of QS from algal and fungal origins

2.10.2. Medicinal plants and phytocompounds as QS inhibitors

Plant and their derived products have attracted interest of scientific community globally in search of novel bioactive compounds including anti-infective compounds. Medicinal plants are widely considered safe and have a long history of use in traditional medicine for the prevention and treatment of infectious diseases. Plant extracts have been reported to acts as inhibitors of QS due to similarity in their chemical structures to those of the AHL and because of their ability to degrade signal receptors or other unkown mechanisms (Vattem et al. 2007; Teplitski et al. 2011). In the last decade there has been an increased interest in search of anti-QS compounds from natural products including plants. QS inhibitors and active compounds have been detected and characterized in various species of plants including pea seedling (Fatima et al. 2010), medicinal plants (Adonizio et al. 2006; Adonizio et al. 2008a; Zahin et al. 2010b); dietry compounds (Vattem et al. 2007), extracts of *Medicago truncatula* seedlings (Gao et al. 2003) and roots of *Panax ginseng*, *Areca catechu* and *Panax notoginseng* (Song et al. 2010; Koh et al. 2011). Aqueous extracts of edible plants and fruits such as *Ananas comosus*, *Musa paradiciaca*, *Manilkara zapota* and *Ocimum sanctum* proved to be QSI against violacein production by *C. violaceum* and pyocyanin pigment, staphylolytic protease, elastase production and biofilm formation abilities of *P. aeruginosa* PAO1 (Musthafa et al. 2010). Extract from *Scorzonera sandrasica* proved effective in inhibiting violacein production in *C. violaceum* (Bosgelmez-Tinaz et al. 2007). AHL degrading abilities have been reported from a large number of legumes — alfalfa, clover, lotus, peas and yam beans (Delalande et al. 2005; Gotz et al. 2007; Fatima et al. 2010). Extracts from different plant parts like leaves, flowers, fruit and bark of *Combretam albiflorum*, *Laurus nobilis*, and *Sonchus oleraceus* were also found to possess anti-QS activities (Schaefer et al. 2008; Al-Hussaini and Mahasneh, 2009). Methanolic extract of fruit of *Capparis spinosa* and *Myristica cinnamomea* have also showed anti-QS activity in various QS systems (Abraham et al. 2011, Chong et al. 2011). Recently, hexane extract of clove bud (*Syzygium aromaticum)* demonstrated significant anti-QS activity against two strains of *P. aeruginosa* (Krishnan et al. 2012). *Lagerstroemia speciosa* attenuates QS-related genes (*las* and *rhl*) and significantly inhibits virulence factors: LasA protease, LasB elastase, and pyoverdin production (Singh et al. 2012).

Garlic extract has been shown to be a potent inhibitor of QS in many studies. It was used in the first human clinical trial of a QSI where 26 cystic fibrosis (CF) patients were administered with garlic or olive oil as placebo. An improvement was observed in lung function, weight and symptom score with the garlic extract (Smyth et al. 2010). The extract was also found to repress QS-controlled expression of *lasB* in a dose-dependent manner in *P. aeruginosa* using a *lasB-gfp* reporter system (Rasmussen et al. 2005a). In the same study, garlic extract was found to alter the architecture of the bacterial biofilms making them more susceptible to tobramycin. *In vitro* and *in vivo* studies have shown a significant inhibition of *P. aeruginosa* QS by crude garlic extract. Persson et al. (2005) identified several active compounds as LuxR antagonists from the garlic extracts. Most significantly cyclic thioacetal (Figure R9A) and cyclic disulfide (Figure R9B) showed only QS activity, while other was toxic to the bacteria. Using bioassay-guided fractionation of garlic extracts, a recent study has implicated ajoene (Figure R9C), a sulfur-rich compound from garlic extract, in inhibiting QS (Jakobsen et al. 2012).

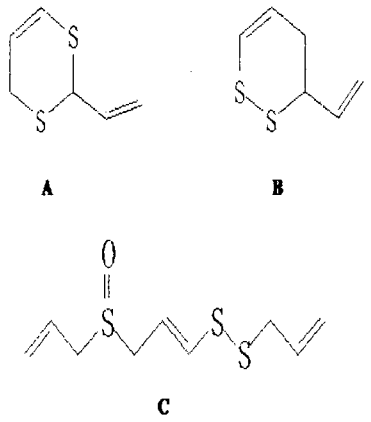


Figure R9. Quorum sensing inhibitors from garlic. A. cyclic thioacetal, B. cyclic disulfide, C. Ajoene

Girennavar et al. (2007 and 2008) reported the study of grapefruit juice and a number of its components for QSI activity. Two types of grapefruit juice, Rio red and Marsh white, were both tested and shown to inhibit AI-1 and AI-2 receptor systems. Two furocoumarins, bergamottin and dihydroxybergamottin (Figure R10) were shown to have strong AI-1 and AI-2 inhibitory activities at concentration as low as 1 $\mu g\ mL^{-1}$. Further investigation showed that both inhibited biofilms in *E. coli* O157:H7, *Salmonella typhimurium* and *P. aeruginosa*, without inhibition of bacterial

growth. Sour orange seeds contain limonoids (Figure R10) such as isolimonic acid, ichangin and deacetyl nomilinic acid 17 β-D-glucopyranoside (DANG) with abilities to cause >90% inhibition of AI-2 activity in *V. harveyi* at 100 μg/ml. Limonoids were also effective against HAI and AI-2 mediated bioluminescence (Vikram et al. 2011). These furocoumarins and limonoids share a furan moiety with the synthetic furanones, which have QSI abilities (Lonn-Stensrud et al. 2007).

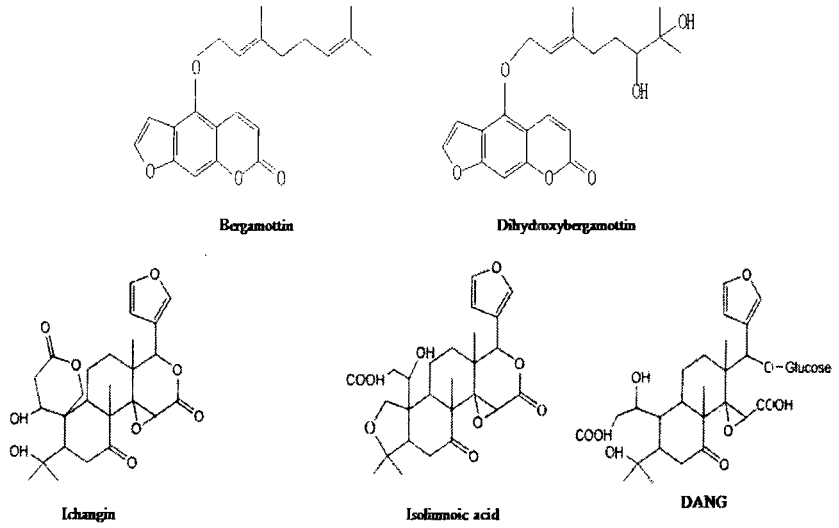


Figure R10. Furocoumarins and limnoids as inhibtors of QS

Park et al. (2008) reported the first example of a natural alkaloid QSI, solenopsin A. Initial investigations in *P. aeruginosa*, showed solenopsin A (Figure R11) to inhibit pyocyanin production, an indication of QS signaling suppression. Competition studies with synthetic AHLs indicated that the rhl signaling system might be the target of solenopsin. Further investigations showed that solenopsin A reduced biofilm production in *P. aeruginosa* in a dose dependent manner, indicating a QS signaling suppression mechanism. Recently, Caffeine (Figure R11) has been shown to inhibit QS regulated violacein production using *C. violaceum* CVO26 biosensor. The phytocompound did not degrade AHL tested rather it inhibited the production of AHL in *P. aeruginosa* PAO1 (Norizan et al. 2013).

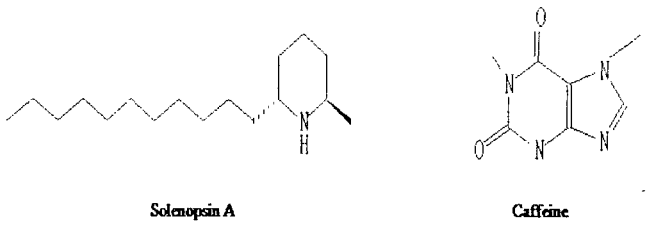


Figure R11. Alkaloids as inhibitors of QS

Polyphenols, best known for their antioxidant properties, have been shown to have an effect on biofilms as reported in a number of cases. Huber et al. (2003) investigated both quorum-sensing inhibition and biofilm inhibition for three compounds (-)-epigallocatechin (EGCG), ellagic acid and tannic acid (Figure R12) using two AHL-dependent quorum sensor strains *P. putida* (pKR-C12) and *E. coli* MT102. EGCG was the most effective in both strains followed by ellagic acid, then tannic acid. Pyrogallol (Figure R12) extracted from medicinal plants such as *Emblica officinalis* and its analogues exhibited antagonism against AI-2 (Ni et al. 2008). Recently, ellagic acid derivatives isolated from *Terminalia chebula* demonstrated inhibition of QS regulated virulence factors in *P. aeruginosa* PAO1 both *in vitro* and *in vivo*. The bioactive fraction showed down regulation of autoinducer synthase and its cognate receptor genes (Sarabhai et al. 2013).

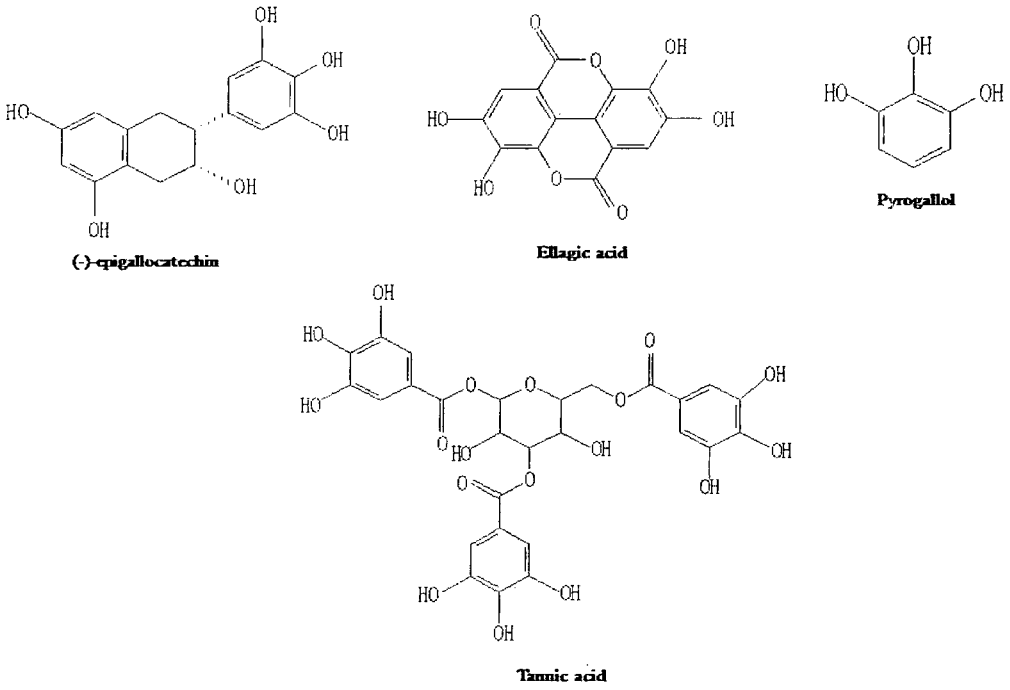


Figure R12. Polyphenols as inhibitors of QS

Flavanoids have been the focus of research for their roles as antioxidant, anti-inflammatory, and anticancer agents. Keeping in view these health benefits, flavanoids such as naringenin, kaempferol, quercetin and apigenin (Figure R13) were evaluated for their QSI activities. All these flavanoids inhibited HAI-1 or AI-2 mediated bioluminescence in *V. harveyi* BB886 and MM32. Quercetin and naringenin were found to inhibit biofilm formation by *V. harveyi* BB120 and *E. coli* O157:H7 (Vikram et al. 2010). Zeng et al. (2008) used computer aided drug design (CADD)

and knowledge of Traditional Chinese Medicines (TCM) to pre-screen potential compounds. Using the crystal structure of TraR from *A. tumefaciens,* five compounds were shown to inhibit biofilm formation in *P. aeruginosa*, the most potent being the flavanoid baicalein (Figure R13). In addition, baicalein was shown to have a marked synergistic effect, retarding biofilm formation when used in combination with the antibiotic ampicillin. Flavan-3-ol catechin (Figure R13), one of the flavonoids from the bark of *Combretum albiflorum* reduces the production of QS-mediated virulence factors-pyocyanin, elastase and biofilm formation by *P. aeruginosa* PAO1 (Vandeputte et al. 2010).

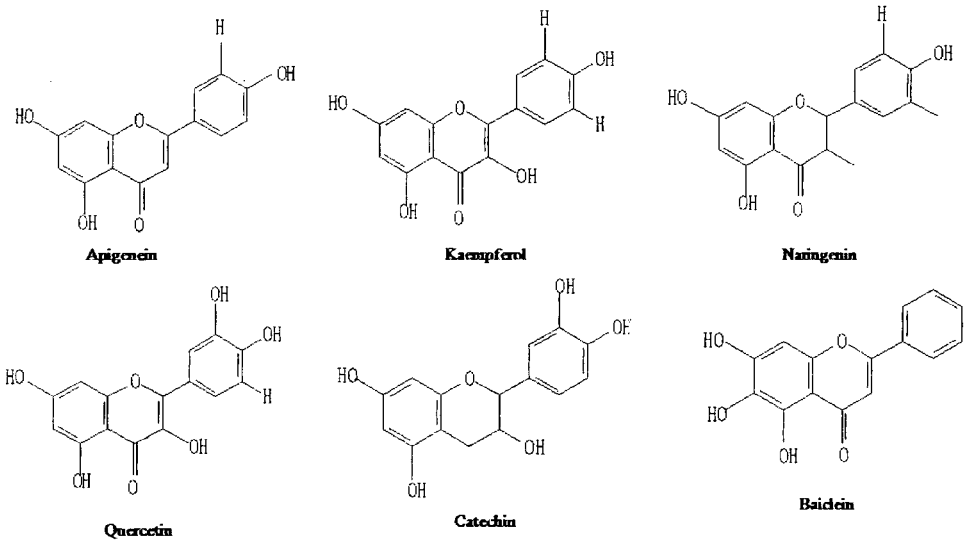


Figure R13. Flavanoids as inhibitors of QS

A new biofilm inhibitor ursolic acid was identified from 13,000 samples of compounds purified from whole plants and separated parts such as fruits, leafs, roots and stems (Ren et al. 2005). Ursolic acid (Figure R14) added at the rate of 10 μg/ml decreased biofilm formation by 79% in *E. coli* and 57-95% in *V. harveyi*, and *P. aeruginosa* PAO1 depending up on the medium. Incidentally, in these experiments, ursolic acid was found to have no effect on QS as observed with *V. harveyi* AI-1 and AI-2 reporter systems (Ren et al. 2005). Hamamelitannin (Figure R14) derived from the bark of the plant *Hamamelis virginiana* inhibits QS in *S. aureus* and *S. epidermidis* at non-inhibitory concentrations. This natural inhibitor of QS has been shown to prevent device associated infections by methicillin resistant *S. aureus* and *S. epidermidis* in a rat graft model (Kiran et al. 2008). *Cuminum cyminum* and its

secondary metabolite methyl eugenol showed significant antibiofilm and antiquorum sensing activity against Gram negative bacterial pathogens (Abraham et al. 2012).

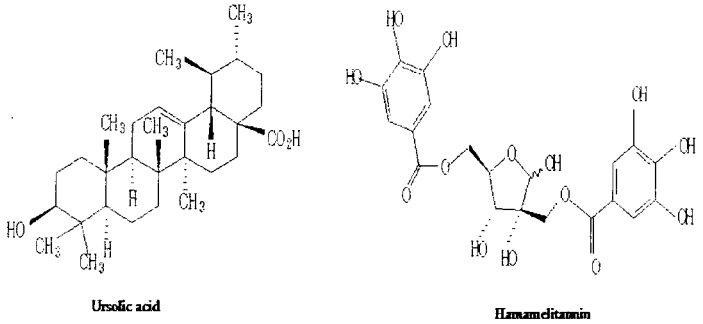


Figure R14. Other phytocompounds as inhibitors of QS

It has been demonstrated that the essential oils from a number of plants can affect quorum sensing regulated functions. Inhibition of quorum sensing signals by oils of rose, geranium, lavender, rosemary has been reported by Szabo et al. (2010). Cinnamaldehyde (Figure R15) and its derivatives affect a wide range of QS regulated activities such as biofilm formation in *P. aeruginosa* and AI-2 mediated QS in different *Vibrio* spp. (Niu and Gilbert, 2004; Niu et al. 2006; Brackman et al. 2008). Kerekes et al. (2013) have reported varying levels of QS inhibitory activity in the essential oils of juniper, clary sage, marjoram and oil components (linalool and terpinene-4-ol) using *Chromobacterium violaceum* assay system. Essential oil component eugenol (Figure R15) has also been reported to inhibit QS controlled gene expression in *P. aeruginosa* and *C. violaceum* biosensors (Zhou et al. 2013).

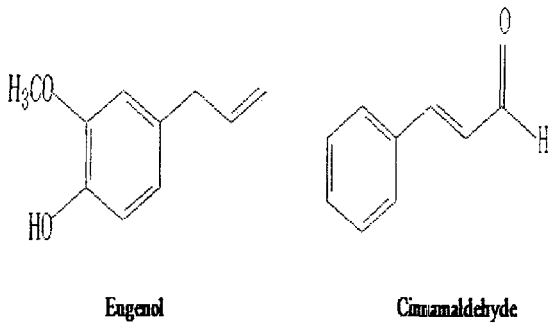


Figure R15. Essential oil components as inhibitors of QS

2.10.3. Antibiotics as QS inhibitors

There is growing awareness that many antibiotics affect multiple modes of bacterial physiology by means that are beyond direct inhibition of currently understood

primary targets of each antibiotic class (Kohanski et al. 2007), and evidence suggests that QS is one alternative target of antibiotics in some species. Therefore, it is worth discussing interactions between antibiotics and bacterial cell-cell communication. In characterizing the outcomes of subinhibitory drug treatments on *P. aeruginosa*, Sofer et al. (1999) were first to demonstrate that erythromycin treatment reduced AHL production. These findings were further elaborated by Tateda et al. (2001), who quantified the reductions of C4HSL and 3OC12HSL to be greater than 70 and 90%, respectively, in PAO1 treated with 2 μg/ml azithromycin (AZM). These reductions coincided with reduced Las- and Rhl-dependent gene transcription and have been corroborated by microarray and proteomic studies (Nalca et al. 2006; Skindersoe et al. 2008). In addition to QS gene misregulation, a reduction in levels of oxidative stress and motility-related genes but an increase in type III secretion (TTS)-related genes were also found. The diminished response to oxidative stress conditions may account for the reduced cell viability in late stationary phase that was reported to occur with increasing concentrations of macrolides (Tateda et al. 2001; Nalca et al. 2006). Likewise, the finding that TTS was induced with exposure to AZM may explain the paradoxical finding that pretreatment of bacterial cultures with macrolides prior to intranasal inoculation in mice led to an enhanced lethal effect (Kobayashi et al. 2002). It is plausible that inducing the TTS system before infection primed the bacteria to enter a virulent state, essentially providing a head start in combating the host's immune response.

Further animal studies have reaffirmed findings that AZM improves *P. aeruginosa* infections. In a cystic fibrosis mouse model (utilizing $Cftr^{-/-}$ mice), treatment with AZM reduced the bacterial load in lungs of mice, and gene expression of the QS regulated *lasB* gene was downregulated *in vivo* (Hoffman et al. 2007). AZM treatment also down regulated production and polymerization of alginate, which combined with decreasing QS responses, was a major contribution to enhanced sensitivity to H_2O_2 and complement and loss of viability in the stationary phase of growth. The benefits of AZM treatments have also been tested in experimental urinary tract infections (UTIs) in mice. Inhibition of motility and biofilm formation by AZM likely contributed significantly to clearance of bacteria from murine renal tissues after 5 days (Bala et al. 2011).

Although azithromycin has shown the highest efficacy in inhibiting QS, other classes of antibiotics could exhibit QSI activities, raising the question as to how these compounds inhibit production of autoinducers. In a screen of 11 antimicrobial compounds, a cephalosporin (ceftazidime [CFT]) and a fluoroquinolone (ciprofloxacin [CPR]) were also able to inhibit AHL production in *P. aeruginosa* and showed regulatory effects very similar to that seen for AZM, as determined by transcriptional profiling after treatment with each antibiotic (Skindersoe et al. 2008). As the structural compositions of AZM, CFT, and CPR are highly divergent, it appears unlikely that each directly blocks activity of AHL synthase proteins and instead seems more probable that a separate, unifying theme for inhibition is at work. It has been suggested that cellular responses to the drugs lead to decreased membrane permeability to block antibiotic penetration, and consequentially this also inhibits autoinducer transfer (Skindersoe et al. 2008). Until this is tested directly, other mechanisms should be considered, including effects on general stress responses and resistance to reactive oxygen species.

Use of antibiotics with QS inhibitors could be effective at blocking bacterial signals that either enhance antimicrobial resistance (e.g., drug efflux) or promote physiological states that enhance persistence (e.g., biofilms). As such, QSI may render cells more susceptible to a variety of antimicrobial compounds. Example of synergism between QSI and antibiotics has also been observed in the regulation of *Staphylococcus* pathogenesis, where the efficacy of the clinically used antibiotics was improved by RNAIII-inhibiting peptide (Balaban et al. 2001, 2003a, b; Giacometti et al. 2003; Giacometti et al. 2005). The antibiotics ampicillin and baicalcein showed synergistic behaviour against *P. aeruginosa* (Zeng et al. 2008). QSIs such as Furanone C30, patulin, penicillic acid and extracts from garlic have been found to be effective in increasing the susceptibility of *P. aeruginosa* to tobramycin, phagocytosis and respiratory burst by polymorphonuclear leukocytes (Hentzer et al. 2003; Rasmussen et al. 2005a; Rasmussen et al. 2005b; Bjarnsholt et al. 2005a, b) and on β-galactosidase activity of *A. tumefaciens* strain NTL4 (Bodini et al. 2009).

Table R3: Some selected inhibitors of quorum sensing

Quorum sensing inhibitor	Organism	Inhibitory activity	Reference
Algae			
Ahnfeltiopsis flabelliformis: α- D-galactopyranosyl-(1 2)-glycerol (floridoside), betonicine and isoethionic acid	*A.tumefacienns*	AHL mediated	Kim et al. 2007
Chlamydomonas reinhardtii: unidentified AHL mimics	*E. coli* (LasRI::lux CDABE)	Bioluminescence	Teplitski et al. 2004
Delisea pulchra: Halogenated Furanone	*E. coli*	Biofilm and swarming, AI-2 signaling system	Ren et a*l.* 2001, Ren et al. 2004,
	P. aeruginosa	Biofilm	de Nys et al. 1993
	S. liquefaciens	Swarming motility	Givskov et al.1996
	V. fischeri	Bioluminescence	
	V. harveyi	Toxin production, Luminescence and Biofilm	Manefield et al. 2000, de Nys et al. 2006
Laminaria digitata: Oxidized halogen HOBr	*Chromobacterium violaceum* CV026	3-oxo-acyl HSLs	Borchardt et al. 2001
Fungi			
Auricularia auricular	*C. violaceum*	Violacein	Zhu et al. 2011
Penicillium: patulin and penicillic acid	*P. aeruginosa*	Biofilm	Rasmussen *et al.* 2005b
Plants			
Arabidopsis exudate: γ-hydroxybutyrate (GHB)	*A. tumefaciens*	AHL signaling	Chai et al. 2007
Camellia sinensis: catechins (Epigallocatechin gallate)	*E. coli*	Transfer of conjugative R plasmid	Zhao et al. 2001
Lotus corniculatus (seedlings)	*A. tumefaciens* NTLR4, *C. violaceum* CV026	Beta-galactosidase Violacein	Delalande et al. 2005

Manilkara zapota	*C. violaceum*	Violacein	
	P. aeruginosa PAO1	Pyocyanin, staphylolytic protease, ealstase and biofilm	Musthafa et al. 2010
Medicago sativa seed exudate L-Canavanine	*C. violaceum*	Violacein	Keshavan et al. 2005
Passiflora incarnate (leaf)	*S. meliloti*	Exopolysaccharide II (EPSII)	
	C. violaceum CV0blu	Violacein	Karamanoli & Lindow, 2006
Pisum sativum (seedlings)	*C. violaceum* CV026	C4HSL-inducible protease, N-acetylglucosaminidase,	Teplitski et al. 2004
		Violacein	Fatima et al. 2010
Raspberry extracts	*C. violaceum*	Violacein	Vattem et al. 2007
Scorzonera sandrasica	*C. violaceum* 12472 and CV026	Violacein	Bosgelmez-Tinaz *et al.* 2007
	Erwinia caratovora	Carbapenem production	
Squash exudate: γ-hydroxybutyrate (GHB)	*A. tumefaciens*	AHL signaling	Chai et al. 2007
Tomato seedlings exudate: γ-hydroxybutyrate (GHB)	*A. tumefaciens*	AHL signaling	Chai et al. 2007
Vanilla planifolia extract	*C. violaceum* CV026	Violacein	Choo et al. 2006
Secondary metabolites			
p-Coumaric acid	*C. violaceum* 5999	Violacein	
	P. chlorophis	Phenazine-1-carboxylic acid (PCA)	Bodini et al. 2009
Salicyclic acid	*A. tumefaciens*	Stimulate AHL-lactonase expression	Yuan et al. 2007
Vanillin	*A. hydrophila*	Biofilm	Choo et al. 2006, Ponnusamy et al. 2009
Manolide, manolide monoacetate and secomanoalide from marine sponge, *Luffariella variabilis*	*E. coli* (LuxR from *V. fischeri*)	Beta -galactosidase and	Skindersoe et al. 2008
	P. aeruginosa	Tetrazolium red	

3.1. List of chemicals used

Chemicals/Biochemicals	Source
Acetic acid (glacial)	Qualigens, India
Acetone	SRL, India
Acetonitrile	SRL, India
Agar	Hi Media, India
Ammonium acetate	Qualigens, India
Ammonium molybdate	Hi Media, India
Azocasein	Sigma-Aldrich Pvt Ltd, India
Benzene	SRL, India
Butanol	SRL, India
N-Butyryl-DL-homoserine lactone	Sigma-Aldrich Pvt Ltd, India
β-mercaptoethanol	SRL, India
Chitin azure	Sigma-Aldrich Pvt Ltd, India
Chloroform	SRL, India
Citric acid monohydrate	Hi Media, India
Disodium hydrogen phosphate	Hi Media, India
Dimethyl formamide	E. Merck, Germany
Dimethyl sulfoxide (DMSO)	SRL, India
Elastin Congo Red	Sigma-Aldrich Pvt Ltd, India
Ethanol	E. Merck, Germany
Ethyl Acetate	SRL, India
Eugenol	Hi Media, India
Ferric chloride	Hi Media, India
Folin Ciocalteu reagent	Qualigens, India
Gallic acid	SRL, India
Glucose	Hi Media, India
Glutaraldehyde	Hi Media, India
N-Hexanoyl-DL-homoserine lactone	Sigma-Aldrich Pvt Ltd, India

Hydrochloric acid	Qualigens, India
p-iodonitro tetrazolium violet	Hi Media, India
Kovac's reagent	Hi Media, India
Magnesium chloride	Qualigens, India
Magnesium sulfate	Qualigens, India
Menthol	Hi Media, India
Methanol	SRL, India
Nitric acid	Qualigens, India
N-(3-Oxododecanoyl)-L-homoserine lactone	Sigma-Aldrich Pvt Ltd, India
O-nitrophenol-β-D-galactopyranoside	Sigma-Aldrich Pvt Ltd, India
Petroleum ether	SRL, India
Phenol (saturated)	SRL, India
Potassium chloride	Qualigens, India
Potassium dichromate	Qualigens, India
Potassium phosphate (dibasic)	Hi Media, India
Potassium phosphate anhydrous	Hi Media, India
Potassium dihyrogen phosphate	Hi Media, India
Sodium carbonate	Qualigens, India
Sodium chloride	Qualigens, India
Sodium citrate	SRL, India
Sodium dihydrogen phosphate	Hi Media, India
Sodium dodecyl sulphate	SRL, India
Sodium hydroxide	Qualigens, India
Sulphuric acid	Qualigens, India
Trichloro acetic acid	SRL, India
Tris HCl	SRL, India
X-gal	Hi Media, India
Zinc chloride	Qualigens, India

Note: Chemicals, which have not been included in the list, were of analytical grade.

3.2. Microbiological media g/l

Basal medium (carbohydrate fermentation) (pH 6.8)

Ammonium dihydrogen phosphate	1.0 g
Potassium chloride	0.2 g
Magnesium sulfate	0.2 g
Phenol red (5% solution)	0.7 ml
Sugar	10.0 g
Distilled water	1000 ml

Brain heart infusion broth

Calf brain, infusion	200.0 g
Beef heart infusion	250.0 g
Proteose peptone	10.0 g
Dextrose	2.0 g
Sodium chloride	5.0 g
Disodium phosphate	2.5 g
Distilled water	1000 ml

King's B agar (pH 7.2)

Proteose peptone	20.0
Dipotassium hydrogen phosphate	1.50
Magnesium sulphate	1.50
Agar	20.0

15mL of glycerol/1000mL of the medium was added.

Luria broth and agar (pH 7.0)

Casein acid Hydrolysate	10.0 g
Yeast extracts	5.0 g
Sodium chloride	5.0 g
Distilled water	1000 ml

*15 g of Agar was added in Luria broth to prepare Luria agar

Muller-Hinton agar (pH 7.3)

Beef extract	300.0 g
Casein acid hydrolysate	17.5 g
Starch	1.75 g
Agar	15.0 g
Distilled water	1000 ml

MR-VP (Methyl red-Voges Proskauer) broth (pH 6.9)

Peptone	7.0 g
Dextrose	5.0 g

Potassium phosphate	5.0 g
Distilled water	1000 ml

Nitrate broth (pH 7.2)

Peptone	5.0 g
Beef extract	3.0 g
Potassium nitrate	5.0 g
Distilled water	1000 ml

Nutrient broth and agar (pH 7.4)

Peptone	5.0 g
Beef extract	1.5 g
Yeast extract	1.5 g
Sodium chloride	5.0 g
Distilled water	1000 ml

*15 g of Agar was added in Nutrient broth to prepare Nutrient Agar

Nutrient gelatin (pH 6.8)

Peptone	5.0 g
Beef extract	3.0 g
Gelatin	120.0 g
Distilled water	1000 ml

Pseudomonas Isolation Agar (Hi-Media, India) (pH 7.0)

Peptic digest of animal tissue	20.0
Potassium sulfate	10.0
Magnesium chloride	20.0
Distilled water	1000 ml

Simmon citrate agar (pH 6.8)

Ammonium hydrogen phosphate	1.0 g
Dipotassium phosphate	1.0 g
Sodium chloride	5.0 g
Sodium citrate	2.0 g
Magnesium sulfate	0.2 g
Bromothymol blue	0.08 g
Agar	15.0 g
Distilled water	1000 ml

Soft agar (pH 7.4)

Peptone	5.0 g
Beef extract	1.5 g
Yeast extract	1.5 g

Sodium chloride	5.0 g
Agar	7.0 g
Distilled water	1000 ml

Starch agar (pH 6.9)

Peptone	5.0 g
Beef extract	3.0 g
Starch	2.0 g
Agar	15.0 g
Distilled water	1000 ml

3.3. Reagents, solutions and buffers

Staining reagents

Crystal violet

Solution A

Crystal violet (90% dye content)	2.0 g
Ethyl alcohol (95%)	20.0 ml

Solution B

Ammonium oxalate	0.8 g
Distilled water	80.0 ml

Gram's Iodine

Iodine	1.0 g
Potassium iodide	2.0 g
Distilled water	300.0 ml

Safranin

Safranin O (2.5% solution in 95% ethyl alcohol)	10.0 ml
Distilled water	100.0 ml

Biochemical test reagents

Barritt's reagent

Solution A

α-napthol	5.0 g
Ethanol (absolute)	95.0 ml

Solution B

Potassium hydroxide	40.0 g
Creatine	0.3 g
Distilled water	100.0 ml

Folin ciocalteu reagent (1/10)

Folin reagent	10.0 ml
Distilled water	90.0 ml

Kovac's reagent

p-Dimethylaminobenzaldehyde	5.0 g
Amyl alcohol	75.0 ml
Hydrochloric acid (concentrated)	25.0 ml

Methyl red solution

Methyl red	0.1 g
Ethyl alcohol (95%)	300.0 ml
Distilled water	200.0 ml

Nitrate test solutions

Solution A

Sulphanilic acid	8.0 g
Acetic acid (5 N)	1000 ml

Solution B

α-Naphthylamine	5.0 g
Acetic acid (5 N)	1000 ml

Solutions and buffers

ECR buffer (pH 7.5)

Tris	100mM
Calcium chloride	1 mM
Sodium citrate buffer (pH 4.8)	0.1M
Tris-HCl Buffer (pH 7.4)	0.05 M
Calcium chloride	0.5 mM
Trichloroacetic acid	10%

Z buffer (pH 7.0)

Disodium hydrogen phosphate	0.06 M
Sodium dihydrogen phosphate	0.04 M
Potassium chloride	0.01 M
Magnesium sulphate	0.001 M
β-mercaptoethanol	0.05 M

Phospahte buffer saline

Disodium hydrogen phosphate	0.02M

Sodium dihydrogen phosphate	0.02M
Sodium chloride	0.6% (w/v)
Sodium carbonate buffer	1 M

M9 buffer

Potassium dihyrogen phosphate	3 g
Disodium hydrogen phosphate	6 g
Sodium chloride	5 g
Magnesium sulphate (1M)	1 ml

3.4. Isolation of bacteria

Samples collected from the JNMCH, AMU, Aligarh and hospital wastewater was suspended in sterile distilled water in the ratio 1:10. Serial dilutions were further prepared in normal saline solution (NSS). From each dilution 0.1 ml of sample was spread onto Nutrient agar medium, as well as selective media such as Pseudomonas isolation agar, King's B agar. Pseudomonas and other Gram negative bacteria were isolated on different culture media, purified and maintained on agar slants at 4°C.

Bacterial isolates were subjected to biochemical tests using standard methods (Cappuccino and Sherman, 1995).

3.5. Biochemical characterization of the isolated bacteria

3.5.1. Grams staining

The Gram staining was done according to the standard procedure of Cappucino and Sherman (1995). The slide was air-dried and examined in oil immersion under a light microscope.

3.5.2. Biochemical tests

Various Gram positive and Gram negative bacterial strains were tested for their biochemical characteristics using standard procedure as described below:

3.5.2.1. Indole test

For the detection of indole production, bacterial culture was inoculated into the tryptone broth and incubated at 37 °C for 24 h. The presence of indole was detected by the addition of Kovac's reagent, which produces a cherry red color in the positive cultures.

3.5.2.2. Methyl red and Voges-Proskauer tests

The culture was inoculated into 5 ml of MR-VP broth and incubated at 37 °C for 24 h. Grown culture were divide into two aliquots (1 ml each) and 3-4 drops of methyl red indicator was added to 1 ml aliquot of the culture. The production of red color was considered as a positive methyl red test. To the other aliquot, 3-4 drops of Barritt's reagent A were added and mixed gently. Immediately, 3-4 drops of Barritt's reagent B were added and mixed again. The development of pink color within 15 min was positive Voges-Proskauer test.

3.5.2.3. Citrate utilization test

This test was done to determine the ability of a bacterial isolate to use citrate as a sole source of carbon. The bacterial culture was streak-inoculated into the Simmon's citrate agar slants and incubated at 37 °C for 24 h. The growth of the culture and development of blue color indicated a citrate positive test.

3.5.2.4. Catalase test

Nutrient agar slants were inoculated with the cultures and incubated at appropriate temperature for 24 h. Catalase production was determined by adding 2-3 drops of H_2O_2 (3%). The instant generation of bubbles or foam indicated a positive test for catalase.

3.5.2.5. Oxidase test

The bacterial culture was streak-inoculated on to nutrient agar. A few drops of freshly prepared oxidase reagent (tetramethyl-p-phenylenediamine dihydrochloride) were added to a filter paper strip. A loopful of the test organism is smeared into the reagent zone of the filter paper. The development of pink, then maroon, and finally blue-black color within 10-15 sec indicated an oxidase positive test.

3.5.2.6. Nitrate test

Five mililitre of nitrate broth was inoculated with the test organism and incubated at 37 °C for 24 h. 3-4 drops of each; solution A (sulphanilic acid) and solution B (α-naphthylamine) were added. The development of pink color indicated a positive test.

3.5.2.7. Starch hydrolysis

Bacterial strains were spot inoculated on the starch agar plates by placing a loopful culture. The plates were incubated at 37 °C for 24 h. Starch in the presence of iodine would impart a blue-black color to the medium, indicating negative result. If the starch has been hydrolyzed, a clear zone of hydrolysis would surround the growth of the organism. This was a positive result.

3.5.2.8. Gelatin hydrolysis

Nutrient gelatin deep tubes were inoculated with the cultures and incubated at the appropriate temperature for 24 h. Following incubation, the cultures were placed in a refrigerator at 4 °C for 30 min. Cultures that remain liquefied produced gelatinase and

demonstrated gelatin hydrolysis. Cultures that solidified on refrigeration lack gelatinase and gave negative reaction.

3.5.2.9. Production of H_2S

All the bacterial strains were streaked on triple sugar iron agar slants and incubated at 37 °C for 24 h. H_2S production was indicated by black precipitate formed along the line of inoculation. Absence of precipitate was evidence of negative reaction. The slants were also examined for acid production and results were recorded as per protocols given by Cappuccino and Sherman (1995).

3.5.2.10. Production of acid and gas from carbohydrates

To detect the production of acid and gas during carbohydrate utilization, Durham's tubes were inserted into the basal broth (in test tubes) in inverted position. Gas production was revealed by the production of bubble inside Durham's tube. Change of the color of the medium from reddish violet to yellow indicated the production of acid by bacteria. These observations were taken after incubation of tubes at 37 °C for 48 h.

3.6. 16S rDNA based identification of selected isolates

The sequencing of 16S rDNA of the strains PAF-14, PAF-79, WAF-38 and WAF-47 was done commercially by DNA Sequencing Service, Macrogen, Inc., Seoul, South Korea using universal primers, 518F (5'CCAGCAGCCGCGGTAATACG3') and 800R (5'TACCAGGGTATCTAATCC3'). Later, nucleotide sequence data was deposited in the Gen-Bank sequence database.

The online program BLAST was used to find out the related sequences with known taxonomic information in the databank at NCBI website (http://www.ncbi.nlm.nih.gov/BLAST) to accurately identify the strains.

3.7. Antibiotic susceptibility test

All the isolated strains were tested for their sensitivity to different antibiotics by disc diffusion method (Bauer et al. 1966). The 0.1 ml of exponentially grown and properly diluted cells was spread on the surface of nutrient agar plates. The antibiotic discs of known potency were then placed on the agar layer using sterile forceps and the plates were incubated overnight at 37 °C. The zone of inhibition of growth around the

antibiotic discs was measured and the results recorded as resistant or sensitive, based on zone sizes. Antibiotic disc usedare listed in table M1.

3.8. Bioassay for detection of AHL production by bacterial cultures

AHL production was detected using biosensor organism, *Agrobacterium tumefaciens* A136 which respond directly to AHLs with carbon chains ranging from C_6 to C_{14} (McClean et al. 2004). Standard method described by McClean et al (1997) for AHL production was adopted.

For bioassay test bacteria were grown on L.B. agar plate. Following overnight incubation the organisms were overlaid with L.B. soft agar containing 100µl of overnight grown culture of *A. tumefaciens* A136 biosensor and 50µl of X-gal solution (20 mg/ml in dimethyl formamide). The biosensor strain themselves were used as a negative control in this assay as both strain lack AHL. The positive test is indicated by a blue coloration due to lacZ expression and X-gal hydrolysis in the *A. tumefaciens* biosensor (Fuqua and Winans, 1996). Negative test for AHLs were indicated by lack of colouration.

3.9. Method for natural AHL extraction from AHL producing isolates

To extract natural AHL from the isolated bacteria method of Shaw et al. (1997) was adopted. Bacterial isolates were grown in LB-medium. The overnight grown culture was centrifuged at 8000 rpm for 20 minutes. The supernatant of the culture medium was filter sterilizeed and the filtrate was extracted with ethyl acetate in a ratio of 7:3 with 0.1% acetic acid. The crude AHL was concentrated in vaccum concentrator at 45°C. The AHL thus obtained was reconstituted in 1 ml acetonitrile and stored at 4°C for future use. The figure M1 depicts the AHL extraction protocol from the over producing strain *C. violaceum* 31532.

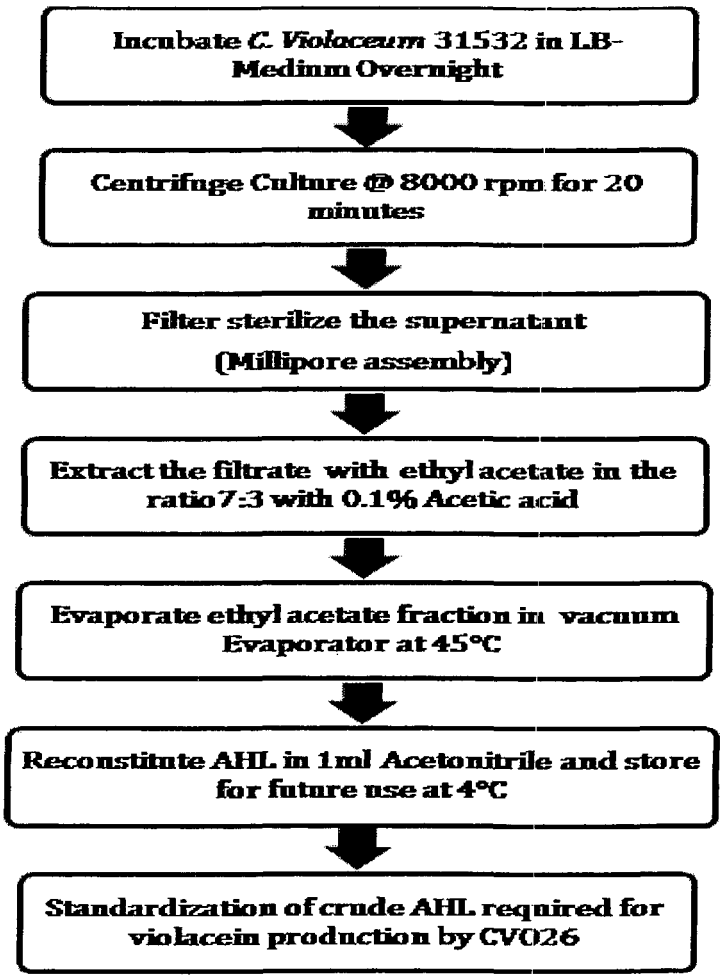


Figure M1. Schematic diagram showing the AHL extraction protocol from *C. violaceum* 31532

3.10. Thin-layer chromatography (TLC) analysis of AHL

TLC was performed on C_{18}reversed-phase plates using a solvent system of methanol/ water (60:40, v/v) essentially as described by Shaw et al. (1997) but using the C. *violaceum* mutant CV026 and *Agrobacterium tumefaciens* A136 as the indicator organism. Synthetic AHLs made up as 10 mM solutions in acetonitrile and extracts of culture supernatants were spotted (2-30 µl) onto glass-backed RP18 reverse phase TLC plates and dried in a stream of cold air. Samples were separated using methanol (60 %, v/v) in water as the solvent. Once the solvent front had migrated to within 2 cm of the top of the chromatogram, the plate was removed from the chromatography tank, dried in air and overlaid with a thin film of biosensor strain seeded in 0-3 % (w/v) LB agar. After overnight incubation at 30 °C, AHLs were located either as purple or blue spots on a white background.

3.11. Mass spectroscopic analysis

High resolution MS was performed as described (Wong et al. 2012) using the using the Waters Synapt G2 HDMS system with the help of direct infusion, carried out at 60 °C, flow rate 0.3 mL/min, with injection volume 20 µL. Mobile phases A and B were 0.1% v/v formic acid in water and 0.1% v/v formic acid in acetonitrile, respectively. The high resolution ESI-MS analysis was performed on a Waters Synapt G2 HDMS system, operated in the ESI-positive mode, with probe capillary voltage set at 3,000 V; desolvation temperature 350 °C; sheath gas 650 L/h; and nebulizer pressure 50 psi. Nitrogen gas was used as the collision gas in the collisionally induced dissociation mode for the MS analysis, with collision energy set at 20 eV. Masslynx software was used to analyze the MS data.

3.12. Detection of virulence factors among AHL producing isolates

3.12.1. LasB elastolytic assay

The elastolytic activity of *Pseudomnas aeruginosa* was determined with the elastin Congo red (ECR; Sigma,) assay (Ohman et al. 1980). A 100 µL aliquot of bacterial supernatant of 16-h culture was added to 900 µl of ECR buffer (100 mM Tris, 1 mM $CaCl_2$, pH 7.5) containing 20 mg of ECR and then incubated with shaking at 37 °C for 3 h. Insoluble ECR was removed by centrifugation, and the absorption of the supernatant was measured at 495 nm. LB medium was used as a negative control.

3.12.2. Pyocyanin quantitation assay

The pyocyanin assay is based upon the absorbance of excreted pyocyanin at 520 nm in acidic solution (Essar et al. 1990). Briefly, 5-ml supernatant from stationary-phase cultures (16 h) in LB broth were mixed with 3 ml of chloroform. The pyocyanin from the chloroform phase was then extracted into 1 ml of 0.2 N HCl, giving it a pink to deep red color, indicating the presence of pyocyanin. The absorbance was measured at 520 nm. Concentration, expressed as micrograms of pyocyanin produced per mL of culture supernatant was determined by multiplying the optical density at 520 nm by 17.072.

3.12.3. Total protease assay

Test strains of *Pseudomonas aeruginosa* were grown overnight in LB-broth. Cells were removed from the medium by centrifugation, and 50-µl aliquots of supernatant

were taken for assay; 500 µl of 0.25% (wt/vol) azocasein (Sigma-Aldrich Ltd. St. Louis, MO USA) in 0.1 M sodium citrate (pH 6) was added to each supernatant aliquot to be tested and incubated at 37°C for 2 h. The protease reaction was stopped, and protein was precipitated, by the addition of 550 µl l of ice-cold 10% (wt/vol) trichloroacetic acid (TCA) followed by incubation on ice for 15 min. Azodye released by the action of proteases in supernatant aliquots was determined at OD_{366} after the removal of precipitated protein by centrifugation (Nithya et al. 2010).

3.12.4. Chitinase assay

Chitinase activity was measured by a modified chitin azure assay as described by Folders et al. (2001). The filter-sterilized supernatants were mixed with sodium citrate buffer (0.1 M, pH 4.8) in 2:1 ratio and 0.5 mg ml^{-1} chitin azure (Sigma). The supernatant chitin azure mixtures were incubated at 37 °C with shaking (200 rpm) for 1 week. The samples were then centrifuged at 15,000 x g for 10 min, and the absorbance at 570 nm was determined. The samples were compared to blanks incubated with medium only.

3.12.5. Extraction and quantification of EPS

Test bacteria were grown in liquid LB medium at 37°C and cells were harvested during late-log-phase by vigorous shaking and centrifugation at 10,500 g for 30 min at 4 °C. The supernatant was filtered through 0.22 µm nitrocellulose membrane filters. Three volumes of chilled 100% ethanol was added to the filtered supernatant and incubated overnight at 4 °C to precipitate EPS (Huston et al. 2004). The precipitated EPS was then quantified by following the method of Dubois et al. (1956).

3.12.6. Swarming motility assay

The method described by Vattem et al. (2007) was used in this assay with slight modifications. Sub-MICs of test agents were mixed with 0.5% LB agar separately and were poured into plates and point inoculated with test strains and incubated at 37 °C for 48 h. The extent of swarming was determined by measuring the diameter of swarm and compared with control.

3.12.7. Assay for biofilm formation

Biofilm formation by the isolated test strains was measured using the polyvinyl chloride biofilm formation assay (O'Toole and Kotler 1998). Briefly, overnight

cultures of test strains were resuspended in fresh LB medium and after 24-h incubation at 30 °C, the biofilms in the microtiter plates were visualized by staining with a crystal violet solution. The plates were rinsed to remove planktonic cells, and the surface-attached cells were then quantified by solubilizing the dye in ethanol and measuring the absorbance at OD_{470}.

3.13. β-galactosidase assay for quorum-sensing signal quantification

β-galactosidase reporter activity was assayed as described by Harjai et al. (2010). Briefly, culture supernatants were extracted by ethyl acetate for quorum-sensing signal molecules (AHLs) from overnight cultures of test bacteria. Then, 2 ml of reporter *E. coli* MG4 (pKDT17) strain and 0.5 mL of the ethyl acetate extracted supernatant was incubated at 30 °C in a water bath for 5 h with rotation at 100 r.p.m. After centrifugation (3200 g for 15 min) of the reporter cell cultures, cell pellet was suspended in an equal volume of Z buffer (Na_2HPO_4 .$7H_2O$, 0.06 M; $NaH_2PO_4.H_2O$, 0.04 M; KCl, 0.01 M; $MgSO_4.7H_2O$, 0.001 M; β-mercaptoethanol, 0.05 M; pH 7.0). To 1 ml of cell suspension, 1ml of Z buffer, 200 µl chloroform and 100 µl of 0.1% sodium dodecyl sulphate was added to lyse cells, and 0.4 mL of O-nitrophenol-β-D-galactopyranoside [4 mg/ml in phosphate buffered saline (PBS)] was also added. Reaction was stopped after the development of yellow colour by the addition of 1ml of 1M Na_2CO_3. OD of the reaction samples was measured at 420 and 550 nm. Units of β -galactosidase were calculated as 1000 x OD_{420} nm-(1.75 x OD_{550nm})/time x volume x OD_{600nm}.

3.14. Collection of plant materials

Traditionally used parts of 37 medicinal plants were obtained from The Himalaya Drug Company Dehradun (Uttarakhand) and or collected /purchased locally. The plant material was identified by plant taxonomist in the Department of Botany, AMU and voucher specimen has been deposited in the Department of Agricultural Microbiology, AMU. Literature search for their traditional uses and known active Compounds are given in table-M2.

Similarly, 25 plant essential oils and some of their ingredients have been purchased from various sources mainly (Wyndmere Natural, USA, The Himalaya Drug

Company Dehradun (Uttarakhand), Aroma sales corporation, New Delhi-India and Dabur India Ltd.). The details of various essential oils is given in table-M3.

3.15. Preparation of plant extracts

Modified method of Alade (1993) was adopted for the preparation of plant extracts. Firstly, the collected plant material was dried in the shade. 100gm of the ground material was soaked in 250 ml of 70% ethanol for 5-6 days and stirred with sterile glass rod after every 18 h. Plant material was filtered using Whatman filter paper No.1. The filtrate was concentrated in a rotatory evaporator (RE-2000A; Associated Scientific Technologies, Delhi, India) at 40°C temperature under vacuum. The concentration was then reconstituted in a known volume of solvent (DMSO) to obtain a crude plant extract for known concentration. All extracts were stored at 4°C for further use.

3.16. Plant screen for quorum sensing inhibition

The standard method of McLean et al. (2004) was adopted to screen plants for their anti-quorum sensing activity. LB agar plates were overlaid with 5 ml L.B. soft agar (containing 0.5% w/v agar) cooled to 45°C which contained 10^6 CFU/ml of the indicator organism *Chromobacterium violaceum* ATCC 12472. Plates were left standing for 30 minutes to let the culture be absorbed. Wells of 8 mm size were punched into agar. Wells were sealed with1-2 drops of molten agar (0.8% agar). The wells were loaded with different concentrations of 100µl of plant extract. Solvent blank was used as negative control. Plant extracts showing anti-QS activity were tested at lower concentrations using disc diffusion method (McLean et al. 2004). About 20µl plant extract of different concentrations were loaded onto the sterile discs and then placed onto the L.B. agar plate and then overlaid with 5 ml L.B. soft agar which contained 100µl of overnight grown culture (containing 10^6 CFU/ml) of the indicator organism *C. violaceum* ATCC 12472 (McLean et al. 2004). A positive QS result was indicated by the lack of pigmentation of the indicator strain in the vicinity of disc impregnated with plant extracts. Similarly, assay was adopted with *Chromobacterium violaceum* CVO26 with the addition of standardized 10µM of C6-HSL.

3.17. Preparation of plant extracts and its fractionation

The plant extract was prepared as described earlier by Aqil et al. (2005). Five hundred (500) g of dry powder of plant material was soaked in 2.5 litre of methanol for 5 days with intermittent shaking and at the end of extraction; the extract was filtered through Whatman filter paper no.1 (Whatman Ltd., England) to make a crude methanol extract. To obtain various fractions of selected plants, the dry powder of plant material (500 g) was further taken for fractionation with the same above procedure with petroleum ether. After extraction, the same material was successively extracted with benzene followed by ethyl acetate, acetone, methanol and ethanol. The filtered extract was concentrated to dryness under reduced pressure on rotary evaporator (RE-2000A, Associated Scientific Technologies, Delhi, India) at 40 °C and stored at 4 °C for future use. Moreover, the yield of solvent dried crude and fractionated extracts was calculated and finally reconstituted in minimum volume of DMSO to perform experiments.

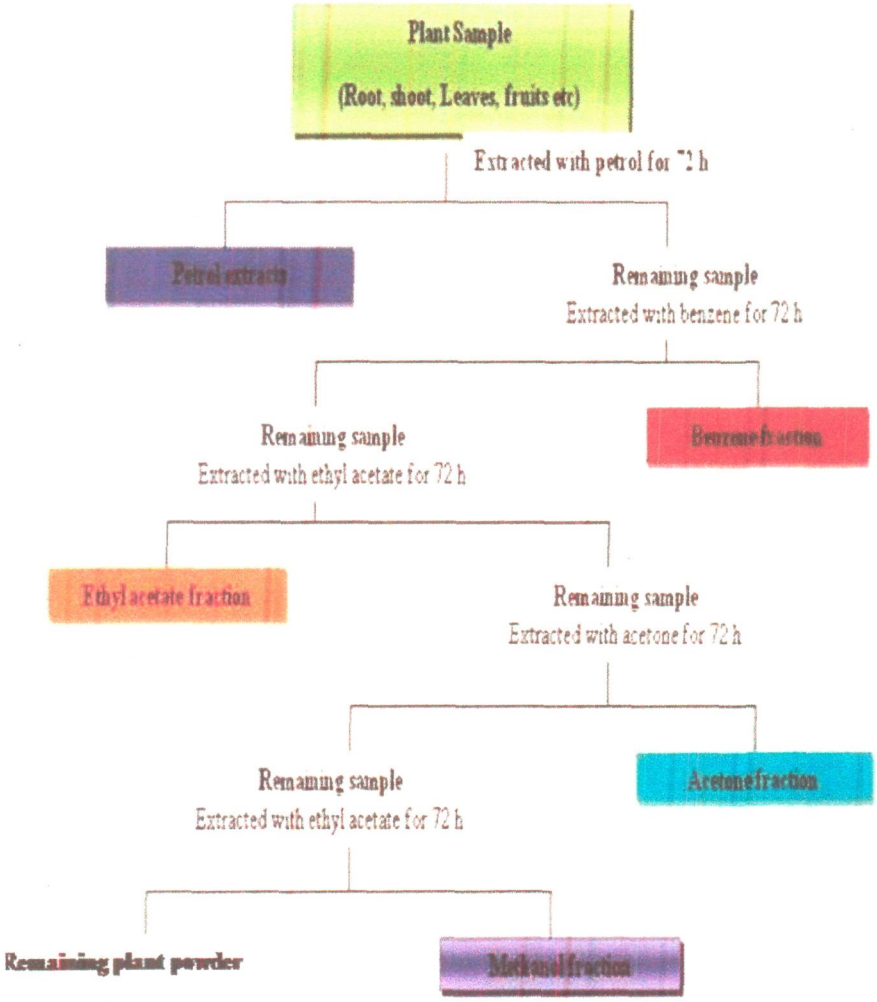


Figure M2. Schematic diagram showing fractionation of plant extracts

3.18. Evaluation of physico-chemical properties of essential oils

Physical and chemical properties of essential oils and active compounds such as colour, refractive index, specific gravity, optical rotation and solubility in alcohol were determined at Flavour and Fragrance Development Centre, Kannauj, India using standard protocols. Colour of oils was noted from physical appearance at 25 °C. Refractive index, extent of optical activity and specific gravity of oils was determined at 27 °C using Abbe's refractometer, polarimeter and specific gravity bottle respectively. Solubility of oil was determined at 27 °C by mixing increment volumes of the oil in specified volumes of the 90% alcohol.

3.19. Screening of essential oils for quorum sensing inhibition

Disc diffusion assay was performed with *C. violaceum* CV12472 to determine the pigment inhibition at the range of sub-MICs of essential oils. Briefly, LB agar plates were spread with 0.1 ml of appropriately diluted freshly grown cultures. Sterile discs (7 mm) impregnated with different amounts of essential oils were mounted. Solvent and Luria broth were used as control. Plates were incubated for 18–24 h at 28 °C to check the inhibition of pigment production around the disc. Growth inhibition, if any, was also recorded. Similarly, assay was adopted with *Chromobacterium violaceum* CVO26 with the addition of standardized 10μM of C6-HSL.

3.20. Determination of minimum inhibitory concentration (MIC)

Minimum inhibitory concentration (MIC) of plant extracts against drug resistant clinical strains was determined by broth dilution method, using specific dye (p-iodonitro tetrazolium violet) as an indicator of growth as described by (Eloff, 1998). Briefly, 2 ml of the plant extract was mixed with 2 ml of Muller-Hinton broth (Hi-Media Ltd., Mumbai, India) and serially diluted into the next tube and so on. 2 ml of an actively growing culture of different test strains was added before incubating for overnight, at 37 °C. After examining turbidity visually, 0.8 ml of 0.02 mg/ml indicator dye (p-iodonitro tetrazolium violet) was added to each tube and incubated at 37 °C. The tubes were examined for the color development, after 30 min. Absence of growth was also confirmed by spreading 0.1 ml of broth from each test tube on normal nutrient agar plates. MIC is defined as the minimum concentration of plant extracts which inhibited the visible growth of test strains.

The MIC values of essential oils were evaluated using broth microdilution assay in sterile 96-well microtiter plates in triplicate (Oroojalian et al. 2010). In each well, 60 µl of LB broth was added. The 60 µl of essential oil was pipetted into the wells in the first column of the plate and two- fold serial dilution was prepared with LB broth. Then 60 µl of the overnight grown bacterial culture was added in each well. Microtiter plates were then incubated at 37°C for 24 hours. After incubation, wells were examined for microbial growth.

The MICs of doxycycline and ceftazidime against the bacterial pathogens were determined using the CLSI macrobroth dilution method (CLSI, 2007). Concentrations below the MICs which did not inhibit visible growth of the test strain were considered as sub-MIC and were further used to study the anti-QS properties.

3.21. Determination of effect of selected test agents on violacein production in *Chromobacterium violaceum* CVO26

Chromobacterium violaceum CV026 was incubated for 16–18 h ($OD_{600\ nm}$ = 0.1) and inoculated to in Erlenmeyer flasks containing Luria broth (LB), LB supplemented with C6-HSL (10µM/l) and LB supplemented with C6-HSL and test agent. The flasks were incubated at 27 °C with 150 rev/min agitation for 24 h in a shaking incubator (Choo et al. 2006).

Extent of violacein production by *Chromobacterium violaceum* (CVO26) in presence of test agents was studied by extracting violacein and quantifying photometrically using method of Blosser and Gray (2000) with little modifications. One-ml culture from each flask was centrifuged at 13000 rev/min for 10 min to precipitate the insoluble violacein. The culture supernatant was discarded and 1 ml of DMSO was added to the pellet. The solution was vortexed vigorously for 30 seconds to completely solubilize violacein and centrifuged at 13000 rev/min for 10 min to remove the cells. Two hundred microlitres of the violacein-containing supernatants were added to 96-well flatbottomed microplates (Polylab, India), four wells per each solution and the absorbance was read with a microplate reader (Thermo Scientific Multiskan Ex, India) at a wavelength of 585 nm. Reduction in the production of pigment in the presence of test agents was measured in terms of % inhibition as, [(OD of control – OD of treated)/OD of control] ×100.

3.22. Determination of effect of test agents on quorum sensing regulated virulence factors

3.22.1. Determination of effect of test agents on LasB elastolytic activity

The elastolytic activity of *Pseudomnas aeruginosa* suspension was determined with the elastin Congo red (ECR; Sigma,) assay (Ohman et al. 1980). A 100 µL aliquot of bacterial supernatant (treated and untreated) of 16-h culture was added to 900 µl of ECR buffer (100 mM Tris, 1 mM $CaCl_2$, pH 7.5) containing 20 mg of ECR and then incubated with shaking at 37 °C for 3 h. Insoluble ECR was removed by centrifugation, and the absorption of the supernatant was measured at 495 nm. LB medium was used as a negative control.

3.22.2. Determination of effect of test agents on pyocyanin production

The pyocyanin assay is based upon the absorbance of excreted pyocyanin at 520 nm in acidic solution (Essar et al. 1990). Briefly, 5-ml supernatant (with or without test agent) from stationary-phase culture of PAO1 (16 h) in LB broth was mixed with 3 ml of chloroform. The pyocyanin from the chloroform phase was then extracted into 1 ml of 0.2 N HCl, giving it a pink to deep red color, indicating the presence of pyocyanin. The absorbance was measured at 520 nm. Concentration, expressed as micrograms of pyocyanin produced per mL of culture supernatant was determined by multiplying the optical density at 520 nm by 17.072.

3.22.3. Determination of effect of test agents on total protease activity

Test strains of *Pseudomonas aeruginosa* and *Aeromonas hydrophila* were grown overnight at the given temperature in LB-broth with or without respective sub-MICs of test agents. Cells were removed from the medium by centrifugation, and 50-µl aliquots of supernatant were taken for assay; 500 µl of 0.25% (wt/vol) azocasein (Sigma-Aldrich Ltd. St. Louis, MO USA) in 0.1 M sodium citrate (pH 6) was added to each supernatant aliquot to be tested and incubated at 37°C for 2 h. The protease reaction was stopped, and protein was precipitated, by the addition of 550 µl l of ice-cold 10% (wt/vol) trichloroacetic acid (TCA) followed by incubation on ice for 15 min. Azodye released by the action of proteases in supernatant aliquots was determined at OD_{366} after the removal of precipitated protein by centrifugation (Nithya et al. 2010).

3.22.4. Determination of effect of test agents on chitinase activity

Effect of test agents on chitinase activity was measured by a modified chitin azure assay as described previously (Folders et al. 2001). The filter-sterilized supernatants were mixed with sodium citrate buffer (0.1 M, pH 4.8) in 2:1 ratio and 0.5 mg ml^{-1} chitin azure (Sigma). The supernatant chitin azure mixtures were incubated at 37 °C with shaking (200 rpm) for 1 week. The samples were then centrifuged at 15,000 x g for 10 min, and the absorbance at 570 nm was determined. The samples were compared to blanks incubated with medium only.

3.22.5. Determination of effect of test agents on EPS production

Test bacteria were grown in liquid LB medium at 37°C and cells were harvested during late-log-phase by vigorous shaking and centrifugation at 10,500 g for 30 min at 4 °C. The supernatant was filtered through 0.22 µm nitrocellulose membrane filters. Three volumes of chilled 100% ethanol was added to the filtered supernatant and incubated overnight at 4 °C to precipitate EPS (Huston et al. 2004). The precipitated EPS was then quantified by following the method of Dubois et al. (1956).

3.22.6. Determination of effect of test agents on swarming motility

The method described by Vattem et al. (2007) was used in this assay with slight modifications. Sub-MICs of test agents were mixed with 0.5% LB agar separately and were poured into plates and point inoculated with *Pseudomonas aeruginosa* strains and incubated at 37 °C for 48 h. The extent of swarming was determined by measuring the diameter of swarm and compared with control.

3.22.7. Determination of effect of test agents on biofilm formation

The effect of sub-MICs of test agents on biofilm formation was measured using the polyvinyl chloride biofilm formation assay (O'Toole and Kotler 1998). Briefly, overnight cultures of test strains were resuspended in fresh LB medium in the presence and the absence of sub-MICs of test agents. After 24-h incubation at 30 °C, the biofilms in the microtiter plates were visualized by staining with a crystal violet solution. The plates were rinsed to remove planktonic cells, and the surface-attached cells were then quantified by solubilizing the dye in ethanol and measuring the absorbance at OD_{470}.

3.23. Light microscopic analysis

Briefly, 1% of overnight cultures of the PAO1, PAF79 and WAF-38 (0.4 OD at 600 nm) were added to 1 ml of fresh LB medium containing cover glass of 1 cm^2 along with and without test agents. After 24 h of incubation, the cover glasses were rinsed with distilled water to remove the planktonic cells and biofilms on the cover glasses were stained with 0.1% crystal violet solution. Stained cover glasses were visualized using light microscope (Nikon Eclipse Ti 100, Japan) (Abraham et al. 2012).

3. 24. Scanning electron microscopy

Biofilms were grown on glass coverslips, in the treated and untreated cultures of test bacteria. After 24 h of incubation, the cover slips were rinsed with distilled water to remove planktonic cells and processed for scanning electron microscopy (SEM) examination as described by Nakamiya et al. (2005) with some modifications. Samples were analyzed by SEM (Hitachi S-3000 N; High Technology Operation, Japan).

3.25. Determination of effect of test agents on β-galactosidase activity

β-galactosidase reporter activity was assayed as described by Harjai et al. (2010). Briefly, culture supernatant was extracted by ethyl acetate for quorum-sensing signal molecules (AHLs) from overnight cultures of PAO1 grown in presence and absence of sub-MICs of test agents. Then, 2 ml of reporter *E. coli* MG4 (pKDT17) strain and 0.5 mL of the ethyl acetate extracted supernatant was incubated at 30 °C in a water bath for 5 h with rotation at 100 r.p.m. After centrifugation (3200 g for 15 min) of the reporter cell cultures, cell pellet was suspended in an equal volume of Z buffer (Na_2HPO_4 .$7H_2O$, 0.06 M; $NaH_2PO_4.H_2O$, 0.04 M; KCl, 0.01 M; $MgSO_4.7H_2O$, 0.001 M; β-mercaptoethanol, 0.05 M; pH 7.0). To 1 ml of cell suspension 1ml of Z buffer, 200 µl chloroform and 100 µl of 0.1% sodium dodecyl sulphate was added to lyse cells, and 0.4 mL of O-nitrophenol-β-D-galactopyranoside [4 mg/ml in phosphate buffered saline (PBS)] was also added. Reaction was stopped after the development of yellow colour by the addition of 1ml of 1M Na_2CO_3. OD of the reaction samples was measured at 420 and 550 nm. Units of β -galactosidase were calculated as 1000 x OD_{420} nm-(1.75 x OD_{550nm})/time x volume x OD_{600nm}.

3.26. Determination of effect of test agents on *Caenorhabditis elegans* survival

The method described by Musthafa et al. (2012) was adopted to study the *in vivo* efficiency of sub-MIC of clove oil in *C. elegans* nematode infection model. Briefly, the young adult nematodes were infected with PAO1 in the 24-well microtitre plate and incubated at 25 °C for 12 h. After incubation, *C. elegans* from the wells were washed thrice with M9 (KH_2PO_4-3g, Na_2HPO_4-6g, NaCl-5g, 1 M $MgSO_4$-1 ml, and Distilled water-1,000 ml) buffer to remove surface-bound bacteria. Around ten infected worms were transferred to the wells of microtitre plate containing 10% LB broth in M9 buffer and incubated at 25 °C with or without 1.6% v/v clove oil treatment. The plate was scored for live and dead worms every 12 h for 4 days. To assess the toxicity if any of the oil, *C. elegans* with clove oil was maintained. Worms were scored for survival by following the method of Moy et al. (2006), in which the plate was shaken by hand and nematode was considered dead if it did not show any muscle tone or movement.

3.27. Phytochemical analysis

3.27.1. Phytochemical analysis of plant extracts

Plant extracts were screened for the presence of major phytocompounds using standard colour test as described below:

Alkaloids: 1 or 2 ml of extract was taken in 5 ml distilled water. Add 2 M HCl until acid reaction, 1 ml of Dragendorff's reagent added. A red or orange precipitate would appear if alkaloids were present (Kapoor et al. 1969; Wagner and Bladt, 1996).

Flavonoids: The method of Kapoor et al. (1969) was used for confirmation of flavonoids. 100 µl extract (100 mg/ml) was dried over a water bath and 5-10 drops of concentrated HCl was added followed by Zn powder. A pink, reddish pink or brown colour developed which indicated the presence of flavonoids.

Glycosides: 100 µl extract (100 mg/ml) was dissolved in distilled water. 1 ml of 1% NaOH solution added. Glycosides gave yellow colour (Odepiyi and Safowora, 1978).

Phenols: 100 µl extract (100 mg/ml) was dissolved in 2 ml distilled water. A few drops of 10% aqueous ferric chloride solution were added. A blue green colour developed which showed the presence of phenols (Fadeyi and Akpan, 1989).

Tannins: 1 ml of extract was taken and a few drops of 5% aqueous ferric chloride were added. If blue black colour appeared and disappeared on addition of dilute H_2SO_4. It indicated the presence of gallo/epitannins while a green brown colour appeared and disappeared on addition of dilute H_2SO_4 indicated the presence of condensed salts of tannins (Segleman and Faransworth, 1969; Bruneton, 1995).

Saponin: A few drops of 1% $NaHCO_3$ solution was added to 100 µl extract (100 mg/ml). Mixture was shaken vigorousy and left for 3 minutes. A honeycomb like froth indicated the presence of saponins (El-Tawil, 1983).

3.27.2. Total phenolic content of plant fractions

The total phenolic content of the plant extract and fractions was determined by the method of Spanos and Wrolstad (1990), as modified by Lister and Wilson (2001). To 0.5 ml of each sample (containing different concentrations (µg/ml) of the extract), 2.5 ml of 1/10 dilution of Folin Ciocalteu reagent and 2 ml of Na_2CO_3 (7.5%, w/v) were added and incubated at 45 °C for 15 min. Each experiment was performed in triplicates. The absorbance of all samples was measured at 765 nm using a UV/Vis spectrophotometer (Spectronic 20 D+, Thermo Scientific, USA). The standard curve of gallic acid is plotted and results were expressed as milligrams of gallic acid equivalent per gram of dry weight (mg GAE/g dw).

3.27.3. IR spectroscopy

The IR-spectra of the active fractions of the plant extracts were recorded on FTIR Spectrophotometer (Shimadzu 8201 PC) with KBr pellets.

3.28. GC–MS analysis of plant extracts and essential oils

The compositions of the extracts/essential oils were analysed by Perkin Elmer GC Autosystem XL and Turbomass with EI source using PE-Wax column (30m x 0.25mm i.d., film thickness 0.25 mm), carrier gas was helium with column head pressure 7 psi connected to data station. Temperature programming: 4 min at 60 °C then using at 4 °C min^{-1} to 200 °C with hold time of 21 min, at 200 °C, split ratio 1:50. The components were identified by comparing their retention times to those of authentic samples, as well as by comparing their mass spectra with those of Wiley 8 and NIST 05 Libraries described by Masada (1976). Quantitative data were obtained by the peak normalisation technique using integrated FID response.

3.29. HPTLC analysis of plant extracts

Test extracts were first diluted ten times in methanol. The test extracts each 2 µL were loaded as 5 mm band length in the 8x10 silica gel $60F_{254}$ TLC plate using Hamilton syringe and CAMAG LINOMAT 5. TLC plates were developed to a distance of 90 mm using the mobile phase toluene: ethyl acetate (7:3 v/v) and chloroform: methanol (9: 1) in a Camag HPTLC twin-trough chamber. After the development time of 20 min, the plate was air-dried for 10 min. These plates were then observed under UV at 254 and 366 nm and the images were documented. Separated bands were quantified by HPTLC densitometric scanning musing Camag TLC Scanner III in the remission–absorption mode at 254 nm operated by Wincats software (version 3.1) to obtain the Rf value and peak areas of the chromatogram (Annegowda et al. 2012)

3.30. Ultra performance liquid chromatography (UPLC) analysis

Extracts from the selected plants namely *Trigonella foenum-graecum, Mangifera indican* and *Psoralia corylifolia* were analyzed for the phenolics on a Shimadzu UPLC system comprised of two LC-20AD-XR pumps, SIL-20A-XR autosampler, and SPD-M20A photodiode array detector (PDA) controlled by Class VP software (ver 7.4, SP3) attached to a Shim-pack XR-ODS-II column (3.0 x 150 mm; 2.2 µ).

A linear gradient of 3.5% phosphoric acid (solvent A) and acetonitrile (solvent B) with flow rate of 0.7 ml/min was used. In the gradient, solvent B was initially 15% for 2 min and increased to 20% for 3 min. The solvent B concentration was further increased to 60% from 3 to 10 min and held for 1 min and returned to 15% for 14 min. The chromatogram was collected from 200 to 600 nm and general phenolics were analyzed at 280 nm.

3. 31. Statistical analysis

All experiments were performed in triplicates and the data obtained from experiments were presented as mean values and the difference between control and test were analyzed using student's *t* test.

Table M1. List of antibiotics used in the study

Antibiotics	Symbol	Disc potency (μg)
Amoxicillin	A	10
Aztreonam	Ao	30
Bacitracin	B	10
Cefepime	(Cpm	30
Cefpirome	Cfp	30
Cefpodoxime	Cpd	10
Ceftazidime	Caz	30
Ceftriaxone	Ctr	30
Cefuroxime	Cxm	30
Cephotaxime	Ce	30
Cephoxitin	Cx	30
Chloramphenicol	C	30
Ciprofloxacin	Cip	5
Clindamycin	Cd	2
Colistin	Cl	10
Doxycycline	Do	30
Erythromycin	E	15
Gatifloxacin	Gf	5
Gentamicin	G	10
Imipenem	I	10
Kanamycin	K	30
Levofloxacin	Le	5
Meropenem	Mr	10
Methicillin	M	5
Nalidixic Acid	Na	30
Nitrofurantoin	Nf	300
Novobiocin	Nv	30
Oxacillin	Ox	1
Piperacillin	Pc	100
Polymixin B	Pb	300 units
Rifampicin	R	5
Sparfloxacin	Sc	5
Streptomycin	S	10
Tetracycline	T	30
Tobramycin	Tb	10
Vancomycin	Va	30

All the antibiotics were purchased from Hi-Media, Mumbai, India

Table M2. Ethnobotanical uses of selected medicinal plants

S. No.	Name of the plants (Family) (Voucher specimen no.)	Common name/ Parts used	Ethnobotanical Uses
1.	*Allium cepa* L. (Liliaceae) (MBD-02/09)	Piyaz/bulbs	The bulbs are acid, sweet, aromatic, thermogenic, antiperiodic, antibacterial, expectorant, diuretic, and tonic. They are useful in haemorrhoids, dysentery, jaundice, pneumonopathy, asthma, bronchitis, opthalmia, vomiting, malarial fever, epilepsy, tumors, wounds, paralysis, leucoderma, and skin diseases (Chopra et al. 1992).
2.	*Allium sativum* L. (Lilliaceae) (MBD-03/09)	Lahsun/bulbs	The bulb contains an antibacterial, anti-inflammatory amoebiasis, oxyuriasis, and colitis. It cures cough, bronchitis and pertusssis. It is also hypocholesterolaemic and thus useful in hypercholesterolaemia and atherosclerosis (Prajapati et al. 2003).
3.	*Acalypha indica* L. (Euphorbiaceae) (MBD-04/09)	Khokli/leaves	Diuretic, cathartric, expectorent, used in snake bit, scabies, rheumatoid, tooth and ear ache (Khare, 2007)
4.	*Agave Americana* L. (Agavaceae) (MBD05/09)	Ban-Kevaraa/leaves	Use to treat ulcer, inflammation, syphilis, tuberculosis and liver disorder (Khare, 2007)
5.	*Areca catechu* L. (Arecaceae) (MBD06/09)	Supari/fruits	Aphrodisiac, Useful in urinary disorder, astringent, anthelmintic, nervine tonic, eminent, in snake bite (Chopra et al. 1992).
6.	*Camellia sinensis* L. Kuntze (Theaceae) (MBD09/09)	Chai/leaves	Astringent, stimulant, gently excitant, diuretic (Brown, 1995)
7.	*Capsicum frutescens* L. (Solanaceae) (MBD-07/09)	Lal mirch/fruits	It is used as a remedy for skin diseases, mild conjuctives and jaundice. Also used to treat inflammations and coughs. The fruit contains a strong stimulant which causes a sensation of warmth when applied to the skin. When taken internally it causes sensation of warmth without any narcotic effect (Prajapati et al. 2003)

8.	*Carum copticum* Benth & Hook (Umbelliferae) (MBD-01/09)	Ajowain/seed	Ajowain seeds contain an essential oil which is about 50% thymol which is a strong germicide, anti-spasmodic and fungicide. It is used in a steeped liquid form against diarrhea and flatulence. In India the seeds are used as a household remedy for indigestion and colic, and used in poultices to relieve asthma and arthritis (Prajapati et al. 2003).
9.	*Cassia fistula* L. (Leguminosae) (MBD-13/09)	Amaltas/fruit	Applied in rheumatism and snake bite, cathartic, emetic, laxative (Chopra, et al. 1992)
10.	*Cichorium intybus* L. (Asteraceae) (MBD-10/09)	Chicory/root	As liver tonic, cardiotonic, diuretic, stomachic, cholagogue, depurative, emmenagogue, hepatomegaly, cephalalgia, inflammations, anorexia, dyspepsia, flatulence, colic, jaundice, splenomegaly, amenorrhea dysmenorrhea and asthma, etc. (Khare, 2007)
11.	*Citrus sinensis* Osbeck (Rutaceae) (MBD- 11/09)	Musambi/Rind	Blood purifier, allays thirst in fever, and improves appetite, also used in billious affection and billious diarrhoea (Chopra et al. 1992).
12.	*Cuminum cyminum* L. (Umbelliferae) (MBD-12/09)	Zeera/seeds	The oil of cumin is used to flavor curries and other culinary preparations, confectionery, beverages, liqueurs, and cordials (Chopra et al. 1992)
13.	*Curcuma longa* L. (Zingiberaceae) (MBD-15/06)	Haldi/rhizomes	It is used in cooking and skin care products. It has wide range medicinal uses. It helps deal with skin problems. Turmeric powder can be used for healing cuts and wounds. It also makes coping with diabetes easier (Prajapati et al. 2003).
14.	*Delonix regia* Raf. (Leguminosae) (MBD-14/09)	Gulmohar/flower	In cooling (Khare, 2007)
15.	*Daucus carota* L. (Umbelliferae; Apiaceae) (MBD-20/09)	Gajar/root	Roasted roots—prescribed in palpitation, burning micturation, cough and bronchitis; increases the quantity of urine and helps the elimination of uric macid; also lowers blood sugar. Juice—a rich source of carotene.
16.	*Emblica officinalis* Gaertn. (Euphorbiaceae) (MBD-16/09)	Amla/fruit	Haemorrhage, diuretic, laxative, in diarrhoae and dysentery (Chopra et al. 1992)

17.	*Ferula assafoetida* Regel (Umbelliferae) (MBD-19/09)	Hing/resin from roots	Asafoetida is known as an antidote for flatulence and is also prescribed for respiratory conditions like asthma, bronchitis and whooping cough. Asafoetida is used mostly in Indian vegetarian cooking, in which the strong onion-garlic flavor enhances many dishes, especially those of Brahmin and Jain castes where onions and garlic are prohibited (Chopra et al. 1992).
18.	*Foeniculum vulgare* Mill.(Umbelliferae) (MBD-17/09)	Saunf/seeds	Feeling and pain in stomach and lower abdomen, hernia pain. Fennel is used to confer flavor and aroma to soups, meat dishes, sausy, bakery and confectionery products, liqueurs and pickles. Fennel oil is used to flavor seasonings, confectionery, culinary preparations, tobacco, cordials and liqueurs (Chopra et al. 1992).
19.	*Hedychium spicatum* Sm (Zingiberaceae) (MBD-22/09)	Kapoor kachri/fruit	Good in liver complaints, vomiting diarrhea, inflammation and pain, in snake bite, as tonic, stomachic (Harborne and Baxter, 1995).
20.	*Hemidesmus indicus* R. Br. (Asclepiadaceae) (MBD-21/09)	Ananthamul/stem	Demulcent, diuretic, in skin diseases, blood purifier, syphilis, rheumatism, scorpion sting, snake bite (Chopra et al. 1992).
21.	*Holarrhena antidysenterica* Wall. (Apocynaceae) (MBD-18/ 09)	Kurchi/bark	Used in dysentery, dropsy, fever, diarrhea and intestinal worm infections. (Chopra et al. 1992).
22.	*Lawsonia inermis* L. (Lythraceae) (MBD-30/09)	Mehndi/leaves	Headache, burning of skin, decoction, used for sore throat. (Chopra, et al. 1992).
23.	*Mangifera indica* L. (Anacardiaceae) (MBD-23/09)	Aam/ leaves	Scorpion sting (Kirtikar and Basu, 1993)
24.	*Nigella sativa* L. (Ranunculaceae) (MBD-25/09)	Kalonji/seed	Stimulant, carminative, diuretic, in puerperal fever, eruptions of the skin, for scorpion sting (Chopra et al. 1992)

25.	*Nyctanthes arbor-tristis* L. (Oleaceae) (MBD-27/09)	Har-singhar/leaves	In fever and rheumatism, chronic fever (Sala, 1994).
26.	*Ocimum sanctum* L. (Lamiaceae) (MBD-28/09)	Tulsi	Diaphoretic, stimulating expectorant, in malaria, in the disorder of genito-urinary disorder (Khare, 2007).
27.	*Piper cubeba* L.f. (Piperaceae) (MBD-29/09)	Kababchini/fruits	The fruits are anti-inflammatory, anthelmintic, carminative, digestive, stomachic, cardiotonic etc. They are useful in asthma, bronchitis, flatulence, dyspepsia, genito-urinary diseases like gonorrhea, cystitis and gleet, rheumatism and hay fever (Prajapati et al. 2003).
28.	*Plumbago zeylanica* L. (Plumbaginaceae) MBD-24/09	Chitra/root	Used in paralytic affection, secondary syphilis, leprosy & Opthalamia (Chopra et al. 1992; Khare, 2007).
29.	*Psidium guajava* L. (Myrtaceae) (MBD-26/09)	Amrud/leaves	Leaves are used for treating diarrhea, coughs, stomachache, toothache and dysentery. Helps in treating indigestion. Used as a skin tonic as well as for treatment of painful menstruation, miscarriages, uterine bleeding and premature labour in women (Prajapati et al. 2003).
30.	*Psoralea corylifolia* L. Papilionaceae; Fabaceae (MBD-37/09)	Babchi/seed	Seed—used in leucoderma, vitiligo, leprosy, psoriasis and inflammatory diseases of the skin, both orally and externally (Khare, 2007).
31.	*Punica granatum* L. (Punicaceae) (MBD-31/09)	Anar/pericarp	The pericarp of the fruit and the bark is used as a traditional remedy against diarrhoea, diabetes, dysentery and intestinal parasites. A decoction of the seed is used treat syphilis. The flower juice is used to treat nose bleeds. Root and stem bark have astringent and anthelmintic properties (Prajapati et al. 2003).
32.	*Sapindus trifoliatus* auct. non L. (Sapindaceae) (MBD-35/09)	Ritha/fruit	Fruit-astringent, emetic,detergent, anthelmintic. Pulp-aqueous solution used as nasal drops in migraine, epilepsy and hysteria. Root-used for gout, rheumatism and paralysis (Khare, 2007).

33.	*Terminalia arjuna* Wight. & Arn. (Combretaceae) (MBD-32/09)	Arjun/bark	Ear ache, tonic, scorpion sting, deobstruent (Chopra et al. 1992).
34.	*Terminalia belerica* Roxb. (Combretaceae) (MBD-36/09)	Bahera/fruit	Stringent, tonic, laxative, antipyretic, in piles, dropsy, diarrhea, leprosy, dyspepsia, headache, astringent, narcotic (Chopra et al. 1992)
35.	*Terminalia chebula* Retz (Combretaceae) (MBD-32/09)	Chabila/fruit	Astringent, laxative, externally in chronic ulcers and wounds and as gargle in stomatitis, diuretic, cardio tonic, useful in carries teeth, bleeding and ulcerations of the gums, ageing, heart ailments and hepatic diseases, etc. (Chopra et al. 1992: Kaur et al. 2002).
36.	*Trigonella foenum-graecum* L. (Leguminosae) (MBD-34/09)	Methi/seeds	The leaves are used for swellings and burns. Seeds are bitter, mucilaginous, aromatic, thermogenic. They are good for fever, vomiting, cough, bronchitis, and colonitis (Chopra et al. 1992).
37.	*Zingiber officinale* Rosc. (Zingiberaceae) (MBD-33/09)	Adrak/rhizomes	Used for flavor in many cuisine. Helps to avoid digestive problems. It is beneficial for coping with cough and cold (Prajapati et al. 2003).

Table M3. List of plant essential oils used

S.No	Scientific Name	Vernacular Name	Part of plant	Major active constituent	Source obtained
1.	*Apium graveolens*	Celery	Seeds	Limonene	Wyndmere Naturals, USA
2.	*Cinnamomum verum*	Cinnamon	Bark	Cinnamaldehyde	Himalaya Drug Co. Dehradun, India
3.	*Citrus limon*	Lemon	Fruit	Citral	Himalaya Drug Co. Dehradun, India
4.	*Citrus paradisi*	Grape	Fruit	α-pinene, limonene	Wyndmere Naturals, USA
5.	*Citrus sinensis*	Orange	Fruit	Limonene	Wyndmere Naturals, USA
6.	*Cymbopogon citratus*	Lemongrass	Leaf	Geraniol	Wyndmere Naturals, USA
7.	*Cymbopogon martini*	Palma Rosa	Leaf	Geraniol	Aroma Sales Corporation New Delhi, India
8.	*Eucalyptus sp*	Eucalyptus	Leaf	Eucalyptol	Aroma Sales Corporation New Delhi, India
9.	*Foeniculum vulgare*	Sweet fennel	Seeds	Transanthole	HiMedia Lab. Mumbai, India
10.	*Lavendula angustifolia*	Lavender	Flowers	Linalool	Wyndmere Naturals, USA
11.	*Mentha piperita*	Peppermint	Leaf	Menthol	Himalaya Drug Co. Dehradun, India
12.	*Myristica fragrans*	Nutmeg	Seeds	Sabinene, α-,β-pinene	HiMedia Lab. Mumbai, India
13.	*Olea europaea*	Olive	Fruit	Oleuropin, tyrosol	Himalaya Drug Co. Dehradun, India
14.	*Petroselinum crispum*	Parsley	Leaf	Myristicin	Aroma Sales Corporation, New Delhi, India
15.	*Rosmarinus officinalis*	Rosemary	Leaf	1,8-cineole, camphor, α-pinene	Wyndmere Naturals, USA
16	*Santalum album*	Sandalwood	Heartwood	Santalol	Wyndmere Naturals, USA
17.	*Syzygium aromaticum*	Clove	flower bud	Eugenol	Wyndmere Naturals, USA
18	*Thymus vulgaris*	Thyme	flower and leaves	Thymol	Dabur India Ltd. New Delhi, India
19.	*Trachyspermum ammi*	Ajowan	Seeds	Thymol	Aroma Sales Corporation New Delhi, India
20.	*Zea mays*	Corn	Seeds	Ionone	Aroma Sales Corporation New Delhi, India
21.	*Zingiber officinale*	Ginger	Kernels	Zingiberene, bisabolene	Wyndmere Naturals, USA

All the essential oils were diluted ten times in 1% DMSO before use in assays.

Table M4. Reference and test microbial strains used

Strains	Relevant genotype	Source
Chromobacterium violaceum ATCC 12472	Wild type	McLean et al. 2004
Chromobacterium violaceum CVO26	mini Tn5 mutant of 31532	McLean et al. 2004
Chromobacterium violaceum 31532	AHL overproducing strain	McLean et al. 2004
Pseudomonas aeruginosa PAO1	Wild type	McLean et al. 2004
Agrobacterium tumefaciens A136	traI-lacZ fusion (pCF218)(pCF372)	McLean et al. 2004
E. coli MG4/pKDT17	*E. coli* DH5a harboring plasmid pMG4/pKDT	Pearson et al. 1994
Isolated bacteria		
PAF1-PAF8, PAF14-PAF26, PAF51, PAF54, PAF79		Clinical (JNMCH, AMU)
PAF9-PAF13, PAF27-PAF35		Soil (hospital wastewater irrigated)
WAF36-WAF50		Hospital wastewater (JNMCH, AMU)

Results

4.1. Isolation, biochemical characteristics and AHL characterization among bacterial isolates

A total of 55 bacterial isolates belonging to genera (*Pseudomonas*, *Klebsiella* and *Aeromonas* sp.) were isolated from clinical and environmental sources on isolation medium used. The isolated bacteria were subjected to biochemical characterization and tentatively identified as shown in table 1a and 1b. These isolates were given a isolate designation and maintained in the laboratory.

Antibiotic sensitivity of the test isolates against 17 antibiotics was conducted using disc diffusion method. On the basis of sensitivity behavior and recommendation of the disc manufacturer, incidence of antibiotic resistance against individual antibiotics was determined (Table 2). The bacterial isolates showed maximum resistance towards aztreonam (60%), followed by amoxicillin, erythromycin (47.2% each), nitrofurantoin, polymixin-B (38.1% each), clinadamycin (36.3%), cefuroxime (32.7%), kanamycin (27.2%), doxycycline (25.4), ceftriaxone (23.6%), cephoxitin (20%), sparfloxacin (16.3%), gentamicin (12.7%), cefpirome (10.9%), ciprofloxacin (9%), imepenem (7.2%) and least resistance was observed against gatifloxacin (05.4%)as depicted in the table 3. Most of the isolates showed resistance to multiple antibiotics. Two isolates of *Pseudomonas aeruginosa* (PAF1 and PAF79) were found to be resistant to 10 antibiotics each, other isolates also showed varied pattern of resistance ranging from combination of 3 to 9 different antibiotics as shown in table 4. The above isolates were further subjected to detection of quorum sensing signal (AHL) production.

Preliminary screening for AHL production by the bacterial isolates was done by cross-streaking them on agar plates against the biosensor *Chromobacterium violaceum* CV026 which produces QS regulated pigment in presence of exogenously produced AHL. Purple pigmentation indicated the presence of short chain AHLs. A total of 29 isolates were found positive for short length (AHL) production, which is indicated by the production of violacein pigment by *Chromobacterium violaceum* CV026 (Table 5; Plate 1). All bacterial isolates were further subjected to *Agrobacterium tumefaciens* A136 based assay to detect the production of quorum sensing signal molecules. This strain is very sensitive and could detect the presence of

long chain AHLs ranging from C6 to C14 types. All the 29 isolates were also found positive for AHL production against *A. tumefaciens* reporter strain (Table 5; Plate 2).

Further, to identify the types of AHLs produced by the isolates. TLC was employed using two biosensor strains, *Chromobacterium violaceum* CV026 and *Agrobacterium tumefaciens* A136. AHLs were extracted from culture broth of test isolates with (700 : 300 supernatant/ dichloromethane). Dichloromethane was removed by rotary evaporation and the residue reconstituted in 1 ml acetonitrile. Identification of AHLs was done by separating extracted AHLs by TLC (C18RP Silica gel plates, Merck, Germany) using a solvent system of methanol/water (60:40, v/v) and subsequent development with biosensor *C. violaceum* CV026. Synthetic AHL standards C4-HSL, C6-HSL were also run simultaneously. Purple colour spots parallel to the position of synthetic standards C4-HSL and C6-HSL (Sigma-Aldrich, USA) on plates were observed. A total of eight *Pseudomonas* isolates showed the presence of C6-HSL while C4-HSL was also detected in all the isolates of *Pseudomonas* producing AHL (Plate 3). Two isolates of *Aeromonas* (WAF38 and WAF47) produced C6-HSL. Since *C. violaceum* CV026 cannot detect AHLs with longer acyl side chains, broad range biosensor, *A. tumefaciens* A136 was employed thereafter for TLC. *A. tumefaciens* A136 contains a plasmid with *traR* promoter and *traG::lacZ* transcriptional fusion. *traG::lacZ* gets activated in presence of exogenous AHL and results in appearance of blue colour. Presence of 3-oxo-C12-HSL was detected in 9 *Pseudomonas* isolates and in the isolate of *Aeromonas* (WAF47) as shown in table 6; plate 4. Unidentified AHL were also detected in *Pseudomonas* isolates PAF2, PAF14, PAF26, PAF79 and WAF47.

In selected isolates, the partially purified AHLs along with standard AHLs were subjected to mass spectroscopic (MS) analysis to confirm the identity of C4-HSL, C6-HSL and 3-oxo-C12-HSL. Mass spectroscopic analysis verified that *P. aeruginosa* PAF14 produced C4-HSL (*m/z* 194.0571) and C6-HSL (*m/z* 222.0997). The results of MS (Figure 1 and 2) confirmed the presence of C4-HSL (*m/z* 194.0571), C6-HSL (*m/z* 222.0997) and 3-oxo-C12-HSL (*m/z* 320.1925) in *P. aeruginosa* PAF79. Similar approach confirmed that *A. hydrophila* WAF38 produced C4-HSL (*m/z* 194.0571) and C6-HSL (*m/z* 222.0997). Further, presence of C6-HSL

(*m/z* 222.0997) and 3-oxo-C12-HSL (*m/z* 320.1925) was verified in *A. aquariorum* WAF-47 (Figure 1 and 2). Table 6 summarizes the MS analyses of these AHLs.

On the basis of above data obtained, four out of 29 AHL producing bacterial isolates were selected for 16S rRNA gene based identification. The partial 16S rDNA sequences of PAF-14, PAF79, WAF38 and WAF 47 were deposited in the GenBank under accession numbers KF813066 (PAF14), JX424425 (PAF79), JX416386 (WAF38) and KF813065 (WAF47). Web-based search and phylogenetic analysis showed that PAF14, PAF79, WAF38 and WAF47 were identified as strains of *Pseudomonas aeruginosa, Aeromonas hydrophila* and *Aeromonas aquariorum* respectively (Figure 3 and 4). All the strains shared 99% similarity in the BLAST search.

Further, all the 29 AHL producing isolates were tested for the presence of quorum sensing regulated virulence factors namely elastase activity, total protease, pyocyanin production, chitinase activity, exopolysaccharide (EPS) production, swarming motility and biofilm formation. *P. aeruginosa* PAO1 was used as positive control for the detection of virulence factor. Among *Pseudomonas* isolates, PAF79 produced all the tested virulence factors strongly. Remaining 26 isolates were also detected to produce varying levels of different virulence factors tested as presented in the table 7. Among the *Aeromonas* isolates, WAF38 was found to exhibit strong production of total protease, EPS and biofilm.

4.2. β-galactosidase activity in AHL producing bacterial isolates

Quantitative induction of β-galactosidase was also estimated in all the AHL producing strains of *Pseudomonas*. The control strain of *Pseudomonas aeruginosa* PAO1 produced 590±21 miller units (MU) of AHL while production of AHLs was found to be variable among the isolates. AHL levels produced by the selected isolates ranged from 124-499 miller units as depicted in figure 5. Highest activity among *Pseudomonas* isolates was recorded in PAF79 (499 miller units) while among the two *Aeromonas* isolates WAF38 produced higher level of AHL (394 miller units).

Based on the highest level of quorum-sensing signal production and on the basis of the virulence factor production, strains *P. aeruginosa* PAF79 and *A. hydrophila* WAF38 were selected for QS interference activity.

4.3. Screening for quorum sensing interference by antibiotics, plant extracts and essential oils

On the basis of isolation and characterization of bacterial isolates for quorum sensing regulated functions (*Pseudomonas aeruginosa* PAF79 and *Aeromonas hydrophila* WAF38) and standard strains *Chromobacterium violaceum* ATCC 12472, *Chromobacterium violaceum* CVO26 and *Pseudomonas aeruginosa* PAO1 were used to screen antibiotics, plant extracts and essential oils/phytocompounds in subsequent sections of the work.

4.3.1. Screening of antibiotics for their violacein inhibitory activity

Interference in purple pigment production in *Chromobacterium violaceum* is indicative of QS inhibition/interference by the antibiotics tested. Thirty three antibiotics disc available for routine sensitivity testing were screened for anti-QS activity (pigment inhibition) against *Chromobacterium violaceum* 12472, of these six antibiotics namely ceftazidime, ciprofloxacin, doxycycline, erythromycin, kanamycin and tobramycin showed varying degree of pigment inhibition in the test strain (Table 8). All other antibiotics exhibited only growth inhibition of the biosensor strain. Azithromycin was used as positive control. As expected, a zone of QS inhibition (halo) was seen with the azithromycin.

All the six antibiotics were further tested against *C. violaceum* CVO26 biosensor strain which does not produce its own AHL but responds to exogenous C6-HSL. CVO26 bioassay was performed using 10 µM synthetic C6-HSL (Sigma-Aldrich, USA). All the six antibiotics demonstrated varying level of AHL mediated violacein pigment inhibition. Doxycycline and ceftazidime produced the highest zone of pigment inhibition in both the biosensor strains as shown in table 8 and plate 5.

4.3.2. Screening of Indian medicinal plants for quorum sensing interference activity

On the basis of traditional uses of medicinal plants and reported bioactivities a total of 37 medicinal plants were selected in this study. Traditionally used parts of these plants were extracted in methanol. The extracts obtained were reconstituted in DMSO and tested at 1000 µg/ ml concentration against *Chromobacterium violaceum* strains by agar well diffusion method. Screening results of methanolic extracts of 37 Indian

medicinal plants for their ability to inhibit/interefere QS regulated violacein production in *Chromobacterium violaceum* 12472 and CVO26 biosensor strains is presented in table 9. In CV12472 test system *Mangifera indica* (leaf) and *Plumbago zeylanica* (root) showed highest zone of pigment inhibition followed by *Camelia sinensis* (leaf), *Psoralea corylifolia* (seed), *Terminalia chebula* (fruit), *Cuminum cymimum* (fruit), *Holarrhena antidysenterica* (bark), *Delonix regia* (flower) and least by *Lawsonia inermis* (leaf). It is interesting to note that methanolic extract of *Trigonella foenum-graceum* seeds enhanced the pigment production by the strain and a dark zone of purple pigment was observed around the well loaded with the extract.

Ten species that showed QS modulation activity in CV12472 were further analyzed using CVO26 biosensor strain. Of these 9 plant extracts reducing violacein in CV12472, extracts of *M. indica* and *P. corylifolia* inhibited the significant pigment production in CVO26 strain. While, the extract of *Trigonella foenum-graceum* (seed) enhanced the pigment production in CVO26 (Table 9). On the basis of strong anti-QS activity in both strains, extracts of *M. indica* (leaf) and *P. corylifolia* (seed) along with *T. foenum-graceum* (seed) which showed the peculiar activity of pigment enhancement, was selected for fractionation based activity determination.

4.3.2.1. Fraction based violacein inhibition/interference activity of plant extracts

Different fractions of *Mangifera indica* (leaves), *Psoralea corylifolia* (seed) and *Trigonella foenumgraecum* (seed) obtained in petroleum ether, benzene, ethyl acetate, acetone and methanol were tested for their QS modulatory activity at varying concentrations against *Chromobacterium violaceum* 12472 (CV12472) strain. Methanol fraction of *Mangifera indica* (leaf) extract showed relatively good anti-quorum sensing activity at 400 µg/ml concentration. At higher concentrations pigment inhibition was accompanied by growth inhibition. Ethyl acetate fraction also exhibited anti-quorum sensing activity at 1800 µg/ml concentration. Pigment inhibition was visible along with growth inhibition in the acetone fraction at 400 and 800 µg/ml while petroleum ether and benzene fractions showed no anti-QS activity at all tested concentrations (Table 10 and Plate 6).

Similarly, fraction based anti-QS activity against *Chromobacterium violaceum* 12472 was demonstrated by *Psoralea corylifolia* methanol extract at 400 and 800 µg/ml concentrations while at 1600 µg/ml pigment inhibition was accompanied by the

inhibition of growth. Similarly, acetone and ethyl acetate extracts also demonstrated comparatively less pigment inhibition accompanied by growth inhibition. However, no activity was detected in petroleum ether and benzene fraction at all tested concentrations (Table 11).

In contrast to the above two plants *Trigonella foenumgraecum* (seed) extract demonstrated pigment enhancement in CV12472 strain. Petroleum ether, ethyl acetate and acetone fractions exhibited antibacterial activity or non significant inhibition while the methanol fraction showed increase in the violacein production by CV12472 strain. At 300 and 600 µg/ml concentrations zone of pigment enhancement was 17 and 20 mm, respectively but no inhibition of growth was observed. At elevated concentration (900 µg/ml) pigment enhancement was visible but was accompanied by a slight zone of growth inhibition (Table 12 and Plate 7).

The study on different fractions of *M. indica*, *P. corylifolia* and *T. foenum-graceum* revealed that the methanol fraction in all the three plants was most active in interfering with the violacein production in CV12472 and CVO26 strains.

4.3.3. Screening of essential oils for violacein inhibition in *Chromobacterium violaceum*

In addition to the above plant extracts, 21 essential oils obtained from authentic sources were also subjected to anti-QS activity against *Chromobacterium violaceum*. QS controlled violacein production was inhibited by four essential oils at tested concentrations. Highest activity in terms of zone of pigment inhibition (18 mm) was recorded in clove oil followed by peppermint oil (13 mm), lavender (12 mm) and least in cinnamon (10 mm) as shown in table 13. Clove oil and peppermint oil demonstrated significant pigment inhibitory activity in CVO26 but lavender and cinnamon oils showed less intense pigment inhibitory property in the strain (Table 13 & Plate 8). No effect on pigment inhibition was observed with other plant essential oils at tested concentrations.

On the basis of the preliminary screening, two antibiotics (Doxycycline and Ceftazidime), three methanolic plant extracts (*Mangifera indica* (leaf), *Psoralea corylifolia* (seed) and *Trigonella foenum-graceum* (seed)) and two essential oils (clove and peppermint) were selected for further studies on the quorum sensing

regulated virulence factors/traits of *Pseudomomas aeruginosa* PAO1 and or other related bacteria

4.4. Determination of minimum inhibitory concentration (MIC) of potential anti-QS agents

To assess the effect of selected test agents (antibiotics, plant extracts and essential oil) on quorum sensing regulated functions, minimum inhibitory concentration (MIC) of the agents was determined against *Chromobacterium violaceum* CVO26, *Pseudomonas aeruginosa* PAO1, *Pseudomonas aeruginosa* PAF79 and *Aeromonas hydrophila* WAF38. The MIC of doxycycline against CVO26, *P. aeruginosa* PAO1, *P. aeruginosa* PAF79 and *A. hydrophila* WAF38 was found to be 8, 16, 64 and 16 µg /ml respectively (table 14). Similarly, ceftazidime displayed MIC values ranging from (0.5-4 µg /ml) while methanol extract of *M. indica* (leaf) showed a wide range of MIC of 750-2000 µg /ml. *P. corylifolia* (seed) extract exhibited MIC of 1250-1500 µg /ml against the test bacteria. The extract of *T. foenum-graceum* exhibited MIC values of 1200 and 2400 µg /ml against PAO1, WAF38 and PAF79 respectively as shown in table 14.

Clove and peppermint oil displayed MIC values ranging from 0.2-6.4% v/v against *Chromobacterium violaceum* CVO26, *P. aeruginosa* PAO1, *P. aeruginosa* PAF79 and *A. hydrophila* WAF38. Eugenol and menthol, the active constituents of these essential oils, demonstrated varying levels of MIC against the test strain as shown in table 14.

4.5. Effect of antibiotics on quorum sensing regulated virulence factors/traits

4.5.1. *Effect of doxycycline on quorum sensing regulated virulence factors/traits*

The extent of inhibition of violacein was determined by the extraction of violacein pigment from CVO26 in presence and absence of doxycycline. Exogenous C6-HSL (10 µM/l) was used for synthesis of violacein. Doxycycline at sub-MICs (1-4 µg/ml) exhibited concentration-dependent pigment inhibitory activity which ranged from 40.4-70%. Bacterial cell count performed on MHA plates at 24h incubation showed no significant difference in the number of colony-forming units (CFUs) in both untreated *C. violaceum* CV026 and *C. violaceum* CV026 treated with doxycycline (Figure 6).

Doxycycline at sub-MICs was tested for its interference against QS regulated virulence factors in *Pseudomonas aeruginosa* PAO1 and a clinical strain PAF79. The sub-MICs (0.5, 1, 2 and 4 µg/ml) of doxycycline showed a concentration-dependent effect on production of virulence factors of *P. aeruginosa* PAO1 as depicted in table 15. However, significant inhibition in the activity of elastase, protease, chitinase, pyocyanin production and swarming motility was not recorded at lower concentration (0.5 µg/ml). Statistically significant percent reduction ($p \leq 0.05$) in pyocyanin production, total protease activity and swarming motility was recorded at 1 and 2 µg/ml. Doxycycline at 4 µg/ml concentration caused maximum percentage decrease in QS-mediated virulence factors such as LasB elastase (67.2%), protease (65%), chitinase (69.8%), pyocyanin (69.1%) and swarming motility (73.1%) in *P. aeruginosa* PAO1 over the untreated control (Table 15 & Plate 9). Similarly, exopolysaccharide (EPS) was extracted from doxycycline treated and untreated cultures of test strains. Spectrometric analysis of the extracted EPS revealed the concentration dependent effect of doxycycline on EPS production. Doxycycline exhibited 30.4 to 69.3 % decrease in EPS production of PAO1 with increasing concentration of sub-MICs tested (0.5–4 µg/ml).

Doxycycline showed a MIC of 64 µg/ml against the clinical strain *Pseudomonas aeruginosa* (PAF79). Therefore, a range of sub-MICs (4, 8, 16 and 32 µg/ml) were selected. Maximum inhibition in the activity of elastase (59.6%), protease (60%), chitinase (74.7%), pyocyanin production (60.5%) and swarming motility (63.6%) was observed at 32 µg/ml doxycycline treatment. A concentration dependent reduction in the above tested virulence factors was recorded as shown in table. However, EPS production was reduced significantly at all tested concentration in PAF79 strain and there was a maximum (69.4%) decrease at 32 µg/ml doxycycline treatment (Table 16).

Doxycycline at above tested concentrations could also significantly inhibit biofilm formation by PAO1 in a concentration dependent manner. Maximum reduction (78.8%) in biofilm forming ability was recorded at highest sub-MIC (4 µg/ml) tested (Table 15). Similarly, significant concentration ($p \leq 0.005$) dependent decrease in biofilm formation was also observed in PAF79 strain when grown in the

presence of sub-MICs of doxycycline. The drug demonstrated a maximum of 77.9 % inhibition over untreated control at the highest sub- MIC tested (Table 16).

The effect of antibiotic was also assessed against selected QS regulated virulence factors/traits of *Aeromomas hydrophila* WAF38. Sub-MICs ranging from 1-8 µg/ml were tested for response against total protease activity, EPS production and biofilm formation. Highest tested concentration (8 µg/ml) was found effective against all the three traits tested. A significant reduction in total protease activity (66.9%), EPS production (51%) and biofilm formation (71.5%) was recorded at maximum (8 µg/ml) tested concentration. At 4 µg/ml concentration significant decrease in total protease activity (56.1%) and biofilm formation (61.9%) was recorded but reduction in EPS production was not statistically significant (Table 17)

4.5.2. *Effect of doxycycline on β-galactosidase activity*

To examine whether anti-QS activity of doxycycline involves well known LasR/RhlR regulators and signaling molecules AHL of QS system, the LasR dependent lasB promoter and reporter gene fusion system (lasB::lacZ) was employed for measuring β-galactosidase activity in cells. The strain PAO1 produced 622 Miller units (MU) AHL in absence of sub-MICs of doxycycline. The levels of AHL decreased significantly to 279 MU at 4 µg/ml concentration. The reduction of β-galactosidase activity in *E. coli* MG4/pKDT17 to 55.1% at 4 µg/ml (Figure 9), showed that doxycycline mediated inhibition of *lasB* promoter activity involves LasR controlled transcription.

4.5.3. *Effect of ceftazidime on quorum sensing regulated virulence factors/traits*

In *Chromobacterium violaceum* CV026, a gradual decrease in the production of violacein content was observed when treated with the increasing sub-MICs of ceftazidime (Figure 10). Maximum of 74.6% inhibition in violacein production was observed at the concentration of 0.25 µg/ml (Figure 10). The reduction in violacein production was also statistically significant at lower concentrations (0.006 and 0.125 µg/ml). No significant reduction in growth of CVO26 was observed at all the tested concentrations.

The effect of ceftazidime in reducing the production of QS-dependent total protease and LasB elastase activity in PAO1 was assessed. The cell-free supernatant

of ceftazidime-treated PAO1 exhibited a significant reduction in the azocasein-degrading protease activity (55.7%), elastin-degrading elastase activity (62.8%), chitinase activity (63.8%), pyocyanin production (61.1%) and EPS production (58.9%) at 0.5 µg/ml concentration over untreated control (Table 18 & Figure 11). The addition of ceftazidime showed a dose dependent decrease in the swarming motility of PAO1 and significant reduction in the migration ability of the pathogen was recorded at all tested concentrations. The maximum inhibition of 81.7% in swarming behavior was recorded at 0.5 µg/ml concentration.

Similar concentration dependent decrease in the QS regulated virulence factors was also observed in the clinical strain of *P. aeruginosa* PAF79. Elastase activity was decreased significantly at all tested concentrations. Ceftazidime (0.25- 2 µg/ml) exhibited 36.8-65.7% reduction in elastase activity as compared to control. Total protease activity, chitinase activity and pyocyanin production was reduced significantly ($p \leq 0.05$) only at 2 µg/ml. EPS production in the test pathogen was decreased by 52.7% and 60.2% at 1 and 2 µg/ml treatment respectively. Swarming ability of the pathogen was also impaired significantly (47.2-76.6%) over control at above tested concentrations (Table 19 & Figure 11).

Significant ($p \leq 0.005$) decrease in biofilm formation was observed in test bacterial strains when grown in the presence of ceftazidime. In PAO1, significanst reduction of 44.4% and 70% was observed at 0.25, 0.5 µg/ml concentration. In a similar manner biofilm forming ability of PAF79 was also reduced significantly at 1 and 2 µg/ml with a maximum of 65.3% reduction in biofilm formation as depicted in table 19 and figure 12.

The effect of ceftazidime was also assessed against *A. hydrophila* WAF38 (Table 20). Concentration dependent decrease in the virulence factors (total protease and EPS production) was recorded. Most effective concentration was found to be 0.5 µg/ml for significant reduction in total protease activity (56.9%) and EPS production (60.2%) was recorded. Biofilm formation in the strain was also reduced considerably ranging from 30.9-65.2% at the tested sub-MICs as shown in table 20.

4.5.4. *Effect of ceftazidime on β-galactosidase activity*

Effect of sub-MICs of ceftazidime on β-galactosidase activity exhibited a concentration dependent decrease. Significant reduction in β-galactosidase activity in *E. coli* MG4/pKDT17 was recorded at sub-MICs. Untreated control produced 538 miller units (MU) where as at 0.03, 0.06, 0.125 and 0.25 µg/ml concentration of ceftazidime 471, 299, 236 and 194 miller units AHL (Figure 13). The reduction in the levels of AHL demonstrates that the inhibition of *lasB* promoter activity involves LasR controlled transcription.

4.5.5. Anti-infective potential of doxycycline and ceftazidime in *C. elegans* nematode model

The anti-infection potential of the sub-MIC of doxycycline and ceftazidime was assessed using a liquid killing assay of *C. elegans* by PAO1 in a 24-well microtitre plate at sub-MICs of antibiotics. Complete (100%) mortality of the *P. aeruginosa* PAO1 preinfected *C. elegans* was observed within 72 h. However, *C. elegans* preinfected with PAO1 and treated with doxycycline (4 µg/ml) and ceftazidime (0.5 µg/ml) separately displayed enhanced survival rate of 55% and 61% respectively (Figure 14).

4.6. Effect of most active fraction of plant extracts on quorum sensing regulated virulence factors

4.6.1. *Effect of methanolic extract of Mangifera indica (leaf) on quorum sensing regulated virulence factors/traits*

The effect of methanol fraction of *M. indica* (leaf) on QS interference activity in terms of reduced violacein production in CVO26 supplemented with synthetic AHL revealed maximum of 83.6% reduction, followed by 55.6%, 32.1% at 8.2% reduction at 800, 400, 200 and 100 µg/ml, respectively, as compared to control (Figure 15). The decrease in violacein production was statistically significant at all tested concentrations except for the lowest concentration (100 µg/ml).

Methanol extract of *M. indica* (leaf) at highest tested concentration (800 µg/ml) caused reduction in elastase (76.2%), total protease (56%), pyocyanin (88.8%), and chitinase (55.3%). At lower concentration (400 µg/ml) significant reduction was also recorded in elastase, total protease activity and pyocyanin

production (Table 21 & Figure 16). Similarly, effect of this extract on *P. aeruginosa* PAF79 was also assessed at sub-MICs (125, 250, 500 and 1000 µg/ml). All tested concentrations demonstrated significant reduction in the activity of elastase (50.8-73.6%), total protease (57.5-82.9%), chitinase (61.1-76.9%) and swarming motility (45.8-70.3%). However, pyocyanin and EPS production was reduced significantly ($p \leq 0.05$) only at 500 and 1000 µg/ml concentration of the extract (Table 22 & Figure 16).

Sub-MICs of *M. indica* (leaf) extract exhibited reduced biofilm formation in PAO1 and PAF79. PAO1 showed significantly decreased biofilm formation at concentrations of 200, 400, 800 µg/ml with a reduction of 48.2%, 55.9% and 72.4%, respectively, as compared to control (Table 21 & Figures 17, 47, 48). In PAF79, biofilm formation was reduced considerably at 1000 µg/ml (74%), moderately at 500 µg/ml and non-significantly at 125 µg/ml (Table 22 & Figure 17).

Effect of the methanol extract on virulence factors of *A. hydrophila* WAF38 is represented in table 23. The extract demonstrated significant reduction in total protease activity (53.9-69.1%) at all tested concentrations. The impact of *M. indica* extract on WAF38 biofilm formation indicated reduction maximally by 82.3% at 1000 µg/ml. At lower concentrations also the reduction was significant ranging from 56.1-76.1%.

4.6.2. *Effect of methanolic extract of Mangifera indica (leaf) on β-galactosidase activity*

The addition of *M. indica* extract decreased β-galactosidase luminescence in *E. coli* MG4/pKDT17 by up to 63.7 % at concentration of 800 µg/ ml (Figure 18), which shows that extract directly inhibits *las*-controlled transcription. Lower concentrations of 200 and 400 µg/ ml too reduced the β-galactosidase activity significantly.

4.6.3. *Effect of methanolic extract of Psoralea corylifolia (seed) on quorum sensing regulated virulence factors/traits*

The inhibitory activity of *P. corylifolia* (seed) extract against bacterial quorum sensing was determined using violacein assay by *C. violaceum* CV026 as depicted in figure 19. The extract exhibited concentration-dependent inhibitory activity with a significant drop in violacein production at all tested concentrations. Maximum reduction of 63.3% over control was observed at 600 µg/ml concentration of the

extract. Bacterial cell count performed on MHA plates at 24 h incubation showed no significant difference in the number of colony forming units (CFU).

Quorum sensing interference by methanol extract of *P. corylifolia* (seed) against *P. aeruginosa* strains is depicted in the table 24, 25 and figure 20. The data showed consistent reduction in LasB elastolytic activity of PAO1 and PAF79 by 49.7 and 46.1 %, respectively. Similarly, total proteolytic assay was reduced by 50.5% in PAF79 and 43.5% in PAO1 at respective sub-MICs. Pyocyanin production was reduced significantly at all concentrations in PAO1. However, in PAF79, pyocyanin production was reduced maximally to 57.8% over untreated control at concentration of 800 µg/ml. Chitinase activity in both the strains was impaired significantly upon treatment with sub-MICs of the extract. The plant extract effectively interfered with the production of EPS in PAO1 and PAF79. Swarming motility was also reduced substantially in both the test strains at respective sub-MICs as depicted in the table 24, 25 and plate 10.

A significant ($p \leq 0.005$) decrease in biofilm formation was observed in test bacterial strains when grown in the presence of *P. corylifolia* (seed) extract (Table 24, 25 & Figure 21, 47, 48). At concentrations of 250, 500 and 1000 µg/ml, the extract showed a significant reduction of 54.1, 70.6, and 79% in biofilm biomass of PAO1. In PAF79 only the highest tested sub-MIC of the extract (800 µg/ml) caused significant inhibition of biofilm (71.6%).

The extract of *P. corylifolia* (100–800 µg/ml) effectively interfered with the QS regulated traits of *A. hydrophila* WAF38 and showed significant reduction in total protease activity to the level of 39.5–65.5 % ($p \leq 0.005$). Similar concentration dependent activity of the extract was observed against EPS production. Dose dependent reduction in biofilm formation *A. hydrophila* WAF38 was observed. Maximum reduction of 50.8% ($p \leq 0.05$) was recorded at 800 µg/ml concentration of the extract as presented in the table 26.

4.6.4. *Effect of methanolic extract of Psoralea corylifolia (seed) on β-galactosidase activity*

Effect of the *P. corylifolia* (seed) extract was also assessed on the β-galactosidase activity of *E. coli* MG4/pKDT17. Dose dependent decrease was recorded for all the

sub-MICs tested and significant reduction of 47.8% was observed at 1000 µg/ml as shown in the figure 22.

4.6.5. *Effects of methanolic extract of Trigonella foenum-graceum (seed) on quorum sensing regulated virulence factors/traits*

Interference of QS by methanol extract of *Trigonella foenum-graceum* at sub-MICs (125, 250, 500 and 1000 µg/ml) was determined using violacein assay in CVO26. The extract exhibited increased production of violacein pigment by CVO26 at all tested concentrations as shown in figure 23. The extract showed a maximum of 74.2% ($p \leq 0.005$) increase in violacein production at 500-1000 µg/ml concentration with no significant difference in the number of colony forming units (CFU).

The extract was further evaluated against QS regulated virulence factors in *P. aeruginosa* PAO1 and clinical strain PAF79. At sub-MICs (125, 250, 500, 1000 µg/ml) the extract showed a concentration-dependent effect on virulence factors of *P. aeruginosa* PAO1 (Table 27). Statistically significant reduction ($p \leq 0.05$) in elastase, total protease, chitinase activity, pyocyanin production and swarming motility was recorded at 500 µg/ml concentration. The extract at 1000 µg/ml concentration caused maximum percent decrease in virulence factors such as elastase (61.3%), protease (59.7%), chitinase (47.5%), pyocyanin (55.7%) and swarming motility (59.7%) in *P. aeruginosa* PAO1 over the untreated control (Figure 24). Exopolysaccharide (EPS) produced by the extract treated and untreated cultures of PAO1 exhibited a maximum of 46.5% decrease over control at 1000 µg/ml concentration.

In the clinical strain PAF79, sub-MICs ranging from 125-1000 µg/ml were selected. Maximum inhibition in the activity of elastase (67.6%), protease (55%), chitinase (87%), pyocyanin production (82.1%) and swarming motility (62.5 %) was observed at concentration of 1000 µg/ml of extract (Table 28 & Figure 24). EPS production was reduced significantly at all tested concentrations and maximum of 77.5 % decrease was observed at highest tested concentration of *T. foenum-graceum* extract.

Statistically significant inhibition of biofilm formation by PAO1 was observed on treatment with sub-inhibitory concentrations of *T. foenum-graceum* extract. The extract demonstrated 24.1-68.7 % decrease in the biofilm forming ability at sub-MICs tested (125-1000 µg/ml) (Table 27 & Figures 25, 47, 48). Similarly, significant

concentration ($p \leq 0.005$) dependent decrease in biofilm formation was also observed in PAF79 strain when grown in presence of sub-MICs of the extract. The methanol extract demonstrated a maximum of 65.5% inhibition over untreated control at the highest sub- MIC tested (Table 28 & Figure 25).

The effect of the extract was also assessed against virulence factors of *A. hydrophila* WAF38. Sub-MICs ranging from 100-800 µg/ml were tested for response against total protease activity, EPS production and biofilm formation. Highest tested concentration (800 µg/ml) was found to be effective against all the three parameters tested with significantly reduced total protease activity (71.6%), EPS production (46.3%) and biofilm formation (76.9%) significantly (Table 29).

4.6.6. *Effect of methanolic extract of Trigonella foenum-graceum (seed) on β-galactosidase activity*

Impact of sub-MICs of *T. foenum-graceum* (seed) extract on β-galactosidase activity of *E. coli* MG4/pKDT17 exhibited a dose dependent decrease. Untreated control produced 768 miller units (MU) where as 623, 557, 483 and 361 miller units AHL production was recorded at 200, 400, 800 and 1000 µg/ml concentration (Figure 26). The reduction in the levels of AHL demonstrates that the inhibition of *lasB* promoter activity involves LasR controlled transcription.

4.6.7. Anti-infective potential of plant extracts in *C. elegans* nematode model

In the absence of plant extracts, complete mortality of the PAO1-preinfected *C. elegans* was observed within 72 h. This result shows the potent pathogenicity of PAO1 towards the *C. elegans* nematode. However, PAO1-preinfected *C. elegans* further maintained with extracts of *M. indica* (800 µg/ml), *P. corylifolia* (1000 µg/ml) and *T. foenum-graceum* (1000 µg/ml) displayed an enhanced survival rate of 72%, 58% and 48% respectively (Figure 27).

4.7. Effect of essential oils on quorum sensing regulated virulence factors

4.7.1. *Effect of clove oil on quorum sensing regulated virulence factors/traits*

Syzygium aromaticum (clove) inhibited violacein production upto 78.4% at maximum sub-MIC (0.12%) tested with little or no significant growth inhibition in *Chromobacterium violaceum* CVO26 (Figure 28). Similarly, a concentration dependent decrease in all the tested QS linked functions was evident in *Pseudomonas*

aeruginosa PAO1 and PAF79 as depicted in table 30 and figure 29. Maximum inhibition of pyocyanin (75%) over control at sub-MICs (1.6%) was recorded in PAO1. Spectrometric analysis of the extracted exopolysaccharide (EPS) revealed that the concentration of EPS decreased with increase in concentration of clove oil. The motility of PAO1 in presence and absence of test concentrations of oil was assessed through swarming motility assay. All tested concentration exhibited significant reduction (52-80%), compared with untreated control (Table 30 and Figure 29).

Clove oil demonstrated a significant reduction in elastase (68.7%) and EPS production (54.8%) at the highest tested concentration (3.2% v/v) in PAF79. Significant reduction in total protease (42.6-69.1%) and swarming motility (23.3-71.6%) was also observed at tested concentrations. Pyocyanin production in the strain was reduced on treatment with sub-MICs of clove oil. Chitinase activity of the PAF79 decreased significantly at 1.6% and 3.2% concentration demonstrating a reduction of 45.4% and 52.7% (Table 31 & Figure 29).

The oil was also tested for its antibiofilm activity against at their respective sub-MICs. Significant ($p \leq 0.05$) decrease in biofilm formation was observed in test bacterial strain when grown in the presence of (0.2-0.8 % v/v) clove oil. At 1.6% concentration, clove oil showed a maximum of 65% ($p \leq 0.005$) reduction in biofilm forming capability of PAO1 (Figure 30 & 46). Similarly, substantial decrease upto 83.3% in biofilm formation by PAF79 was observed at maximum tested sub-MIC (3.2% v/v) concentrations of clove oil.

Further, effect of sub-MICs of clove oil on virulence factor of *Aeromonas hydrophila* is presented in table. The result demonstrated a significant decrease in total protease (57%), EPS production (71%) at highest tested sub-MIC value. Similarly, in biofilm quantification assay, a concentration dependent decrease in biofilm formation was observed in the test bacteria when treated with oil at varying concentrations. The oil showed 35-66% reduction in biofilm of *A. hydrophila* at concentrations of 0.05–0.4% v/v (Table 32).

4.7.2. *Effect of clove oil on β-galactosidase activity*

The treatment of PAO1 with 1.6% v/v clove oil significantly reduced the AHL levels (337 miller units) as compared to untreated control (770 miller units). Thus 56 %

reduction of β-galactosidase activity in *E. coli* MG4/pKDT17 was achieved (Figure 31), indicating that clove oil mediated inhibition of *lasB* promoter activity involves LasR-controlled transcription.

4.7.3. *Effect of Mentha piperita (peppermint oil) on quorum sensing regulated virulence factors/traits*

Anti-QS property of *Mentha piperita* (peppermint) oil was firstly assessed for pigment inhibition in CVO26. The result of the violacein quantification assay is depicted in figure 32. The sub-MICs of peppermint oil exhibited concentration-dependent violacein inhibitory activity at all tested concentrations. At highest concentration tested, maximum reduction of 83.3% was recorded. Bacterial cell count performed on MHA plates at 24 h incubation showed no significant difference in the number of colony-forming units (CFUs) between untreated *C. violaceum* CVO26 and C. *violaceum* CVO26 treated with sub-MICs of the oil (Figure 32).

The effect of sub-MICs of the oil in reducing the production of QS-dependent virulence factors in PAO1 and PAF79 is presented in table and figure. Significant decrease in pyocyanin production (52.4-85.2%), EPS production (39.9-76.52%) and swarming motility (50.6-81.3%) of PAO1 was recorded at sub-MICs of the oil tested. Sub-MICs (0.75-3%) were found effective in inhibiting activity of elastase, total protease and chitinase to significant levels (Table 33 & Figure 33). Similar results were obtained for PAF79 treated with sub-MICs (0.2-1.6%) of peppermint oil. Concentration dependent effect of the oil was observed in all the virulence factors/traits tested. Maximum reduction of (87.3%) was recorded in pyocyanin production followed by elastase activity, chitinase activity, swarming motility, EPS production and total protease activity at the highest tested concentration (3%) over control (Table 34 & Figure 33).

Anti-biofilm activity of the test oil was investigated against PAO1 and PAF79. The data presented in table 33, 34 and figure 34 showed the quantitative analysis of biofilm inhibition in PAO1 and PAF79. Addition of respective highest sub-MICs of oil led to a dose dependent reduction in biofilm formation by upto 84% and 88.1% in PAO1 and PAF79, respectively.

The oil of peppermint (0.1–0.8% v/v) effectively interfered with the QS system of *A. hydropila* WAF38 by significantly reducing the total protease activity to the level of 24.5–71% ($p \leq 0.005$) and EPS production by 39-77.9%. Maximum decrease (74.8%) in biofilm formation at 0.8% v/v concentration of the oil was observed as depicted in table 35.

4.7.4. *Effect of Mentha piperita (peppermint oil) on β-galactosidase activity*

To exclude the influence of other QS systems in *P. aeruginosa*, *E. coli* MG4/pKDT17 that produces LasR and contains the lasB promoter fused to lacZ was used. The addition of peppermint oil decreased significant β-galactosidase activity in *E. coli* MG4/pKDT17 by up to 41.9% and 54.5% at 1.5 and 3% v/v (Figure 35), which shows that peppermint oil directly inhibits las-controlled transcription.

4.7.5. Anti-infective potential of essential oils in *C. elegans* nematode model

The anti-infection potential of the sub-MIC of clove oil and peppermint oil was assessed using a liquid killing assay of *C. elegans* by PAO1 in a 24-well microtitre plate. Complete mortality of the *P. aeruginosa* PAO1 preinfected *C. elegans* was observed within 72 h. However, *C. elegans* preinfected with PAO1 further treated with clove oil (1.6% v/v) and peppermint oil (3% v/v) displayed enhanced survival rate of 62% and 67% (Figure 36). However, clove oil alone demonstrated no significant mortality of *C. elegans* at tested concentrations.

4.8. Effect of essential oil compounds on QS regulated virulence factors

Two pure phytocompounds which are the known active constituents of clove oil and peppermint oil were also evaluated for their effect on QS regulated factors in *Chromobacterium violaceum* CVO26, *Pseudomonas aeruginosa* PAO1, PAF7 and *Aeromonas hyrophila* WAF38.

4.8.1. *Effect of eugenol on quorum sensing regulated virulence factors/traits*

Eugenol exhibited a concentration dependent decrease in QS regulated violacein production and statistically significant inhibition was recorded at all tested concentrations (Figure 37). Maximum reduction of 80% was recorded at 1% v/v concentration while lowest of 41% decrease over control was observed at 0.1% v/v eugenol concentration without any significant decrease in growth of the bacteria.

Further, effect of eugenol on QS regulated functions of *Pseudomonas aeruginosa* PAO1 and PAF79 was determined. Significant reduction ($p \leq 0.001$) in elastase (47-82%), total protease activity (44-87%) and pyocyanin production (45-85%) of PAO1 was recorded at tested sub-MICs (0.1-0.8% v/v) of eugenol. Chitinase activity got lowered significantly (39.8-63.2%) at sub-MICs ranging from 0.2-0.8% v/v eugenol. Similarly, decrease in EPS production was also dose dependent but significant reduction (49.1%) was observed at 0.8% v/v concentration. However, swarming migration in PAO1 was not inhibited significantly at any of the tested sub-MICs of eugenol (Table 36 & Figure 38).

Eugenol demonstrated significant inhibitory activity on total protease (61.1-91.3%), pyocycanin production (31.1-82.2%) and EPS production (31.3-69.8%) of PAF79 at sub-MICs tested. Similarly, elastase and chitinase activity was also inhibited in concentration dependent manner and maximum inhibition of 75.2% and 72.8% was recorded respectively, at highest sub-MIC of eugenol. Similar to the result obtained with PAO1, none of the concentrations tested could inhibit the swarming motility of PAF79 to statistically significant levels. However, biofilm formation in both the strains of *P. aeruginosa* was reduced considerably upon treatment with sub-MICs of eugenol as presented in the table 37 and figure 38.

Eugenol demonstrated dose dependent reduction in the total protease activity and EPS production by A. *hydrophila* WAF38 also. No significant decrease in total protease or EPS production was observed at the lowest tested concentration of eugenol (0.075% v/v). Total protease activity was reduced by 43.2-64.2% at sub-MICs (0.15-1.5% v/v) of eugenol. Similar, significant decrease in EPS production by 43.6-69.9% was recorded at 0.15-1.5% v/v concentrations (Table 38). Similarly, biofilm formation by WAF38 was reduced upto 84% by eugenol (0.15-1.5% v/v).

4.8.2. *Effect of eugenol on β-galactosidase activity*

Effect of eugenol on AHL production was also assessed using β-galactosidase assay in *E. coli* MG4/pKDT17. Untreated PAO1 produced 590 Miller units of AHL where as at 0.1%, 0.2%, 0.4% and 0.8% of eugenol, quantity of AHL produced was 551, 474, 403 and 244 miller units, respectively. The results obtained demonstrated a significant decrease of 31.6% and 58.6% at 0.4 and 0.8% eugenol concentration (Figure 40).

4.8.3. *Effect of menthol on QS regulated virulence factors/traits*

Menthol exhibited a concentration dependent decrease in QS regulated violacein production. Maximum reduction of 85% was recorded at 400 µg/ml concentration while lowest of 26% decrease over control was observed at 50 µg/ml menthol concentration (Figure 41).

Effect of menthol on QS regulated virulence factors of *Pseudomomas aeruginosa* PAO1 and PAF79 revealed a concentration dependent decrease in all the functions. Highest reduction in all the virulence factors was observed at 800 µg/ml in PAO1. Decrease in total protease activity was highest (84.2%) followed by pyocyanin production (83.5%), elastase activity (78.7%), swarming motility (78%), EPS production (57.7%) and least in chitinase activity (54.6%) as presented in table 39 an figure 42.

Similar dose dependent decrease was recorded in the virulence factors of PAF79 as depicted in the table 40 and figure 42. Maximum reduction in activities of elastase (80.9%), total protease (68.3%), chitinase (51.1%), pyocyanin production (88%), EPS production (65.7%) and swarming motility (69.2%) was observed at highest sub-MIC (400 µg/ml).

A significant decrease in biofilm formation was observed in test bacterial strains when grown in presence of menthol. Highest reduction (80%) in biofilm formation of PAO1 was observed at 800 µg/ml concentration followed by 57 and 23% reduction at 400 and 200 µg/ml, respectively. Similarly, menthol (50-400 µg/ml) effectively interfered with the biofilm formation of *P. aeruginosa* PAF79 and showed significant reduction (77.4%) in biofilm forming ability at highest sub-MIC tested (table 40 & figure 43).

In *Aeromonas hydrophila* WAF38, menthol inhibited total protease significantly (52.5%) at 200 µg/ml while at lower concentrations reduction observed was not statistically significant. EPS produced by untreated *A. hydrophila* WAF38 was lowered significantly (58.3-66.6%) at sub-MICs (50-200 µg/ml). Similarly, biofilm formation was also reduced considerably ranging from 27.9-80% over untreated control at sub-MICs of menthol tested (Table 41).

4.8.4. *Effect of menthol on β-galactosidase activity*

E. coli MG4/pKDT17 that produces LasR and contains the lasB promoter fused to lacZ was used to exclude the influence of other QS systems in *P. aeruginosa*. The addition of menthol decreased β-galactosidase luminescence in *E. coli* MG4/pKDT17 by up to 60% at 800 μg/ml, which demonstrates that menthol directly inhibits las-controlled transcription (Figure 44).

4.8.5. Anti-infective potential of eugenol and menthol *in C. elegans* nematode model

The anti-infection potential of the sub-MIC of menthol was assessed in *C. elegans* animal model by PAO1 in a 24-well microtitre plate. Complete mortality of the *P. aeruginosa* PAO1 preinfected *C. elegans* was observed within 72 h. However, *C. elegans* preinfected with PAO1 further treated with eugenol (0.5% v/v) and menthol (800 μg/ml) displayed enhanced survival rate of 71% and 58% respectively (Figure 45).

4.9. Phytochemical analysis of plant extracts

On the basis of promising QS interference activity of the three plant extracts, they were subjected to determination of total phenolics contents in all fractions obtained as presented in table 43. The total phenolic content in various fractions ranged from 32.3-538.2 mg GAE/g of dry extract. Futher major groups of phytocompounds were determined in active fractions by color test and infrared spectroscopy (IR) analysis as shown (table 42 & figure 51, 52, 53). Similarly, GC-MS of the active fraction was also analyzed

4.9.1. Phytochemical analysis of *Mangifera indica* (leaf) extract

Phytochemical analysis of *Mangifera indica* extracts revealed the presence of tannins, glycosides and phenolics as major groups of compounds. The total phenolic content (mg GAE/g) of extract and various fractions determined by the Folin-Ciocalteu method showed highest polyphenolic content (538±3.4) in acetone fraction followed by methanol, ethyl acetate, benzene and least in petroleum ether fraction (Table 42a). GC-MS analysis of the most active fraction revealed the presence of 16 components using direct similarity search for *M. indica*. The compounds identified were 1,2,3-Benzenetriol (15.6%), Benzoic acid, 4-hydroxy (12.09%), n-Hexadecanoic acid

(9.96%), 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl (8.48%) as evident from the GC-MS spectra (Table 44 and Figure 59). These numbers may be extended with the help of chemo metric techniques.

4.9.2. Phytochemical analysis of *Psoralea corylifolia* (seed) extract

Phytochemical analysis of *P. corylifolia* (seed) extract revealed the presence of alkaloids, phenolics and tanins as major group of compounds. The total phenolic content of various fractions (mg/g of dry extract) was determined as gallic acid equivalent by the Folin-Ciocalteu method. Methanol fraction of seed contained 367.6±1.5 mg GAE / g of dry extracts followed by acetone (337.6±1.4), ethyl acetate (292±2.3), benzene (43.3±1.1) and pertroleum ether (43.1±1.0) fractions as depicted in table 43.

A total of 21 chemical components were identified in seed extract by GC-MS analysis. These numbers may be extended with the help of chemo metric techniques. The major compounds identified were 9,12-Octadecadienoic acid (35.72%) followed by 4-[3,7-Dimethyl-3-vinyl-1,6-octadienyl]phenol (27.73%), Palmitic acid (23.12%), Myristic acid (1.050%), The remaining compounds were present in percentages of 0.1 to 0.5 as depicted in table 45 and figure 61.

4.9.3. Phytochemical analysis of *Trigonella foenum-graceum* (seed) extract

Phytochemical analysis of fractions revealed the presence of alkaloids, phenolics, tanins and glycosides as major group of compounds. The total phenolic content (mg/g) of *Trigonella foenum-graceum* various fractions showed highest polyphenolic content (199.8±2.3) in methanol fraction followed by acetone (132.3±1.5), petrol ether (146.4±6.3), benzene (77.7±0.65), ethyl acetate (65.4 ±1.9) and petroleum ether (56±1.1) fractions as depicted in table 43.

A total of 18 chemical components were identified in seed extract by GC-MS analysis. These numbers may be extended with the help of chemo metric techniques. The major compounds identified were 1,3,7-Trimethyl-3,7-dihydro-1h-purine-2,6-dione (40.82%) followed by Methyl 14-methylpentadecanoate (8.22%), Palmitic acid (6.41%), 1,2,3-Benzenetriol (6.13%), Linoleic acid, methyl ester (5.58%) and Capric acid (4.2%). The remaining compounds were present in percentages of 2.01 to 0.1 as depicted in table 46 and figure 61.

4.9.4. HPTLC analysis of methanol fractions of most active plant extracts

To identify the major compounds to be used as marker for standardization of plant extracts, the most active fraction of *Mangifera indica, Psoralea corylifolia* and *Trigonella foenum-graceum* were subjected to HPTLC analysis using two solvent systems (toluene-ethyl acetate and chloroform-methanol), visualizing and scanning at 254 and 366 nm. The results presented in figure 54 and 55, indicate the presence of large number of compounds. Developed chromatograms were first inspected under UV-254 and then at UV-365 nm. At 254 nm quenching zones are indicative of the compounds conjugated with double bonds e.g. anthraglycosides, arbutin, coumarins, flavonoids and some alkaloid types. Fluorescent zones detected at 365 nm suggest for the presence of all anthraglycosides, coumarins, flavonoids, phenolcarboxylic acids and some alkaloids types.

4.9.5. Ultra Performance Liquid Chromatography (UPLC) analysis of methanol fractions of most active plant extracts

Extracts of the *Mangifera indica, Trigonella foenum-graceum* and *Psoralea corylifolia* were analyzed by UPLC monitoring the chromatogram at 280 nm for general polyphenolics. In the absence or lack of standards reference compounds, we analyzed the extracts in a range of 200 to 600 nm to understand major phytochemicals (Figure 56-58). No peaks at higher wavelength were found corresponding to anthocyanins in any of the plant extracts. However, various peaks were detected at 280 nm, suggesting the presence of various phenolic compounds.

In the extract of *Mangifera indica* various peaks were detected at 280 nm (Figure 56c); a total of six peaks being the major ones. In the UPLC analysis, the retention time of the peaks were found 5.93, 8.1, 8.3, 10.05, 11.9 and 12.45 min. Further analysis of these peaks in the photo-diode array detector, we found the maximum absorbance at 310 nm (peak 1), 290 (Peak 2), 212 and 273 (peak 3), 238, 260, 319 and 373 (peak 4), 303 (peak 5) and 273 (peak 6) further suggesting the phenolic origin of the peaks.

Similarly, four and six peaks were detected in *Trigonella foenumgraecum* and *Psoralea corylifolia* respectively (Figure 57c). In *T. foenumgraecum*, largest peak was found at 7.8 min (peak 2; λ max-271) followed by peak 1 (Rt 4.3 min; λ max-217 and

271), peak 3 (Rt 4.3 min; λ max-270), and peak 4 (Rt 12.35 min; λ max-272) (Figure 58c). *Psoralea* showed maximum number of peaks and a total of 6-10 major peaks were detected. Interestingly, all of the peaks are eluted in between 6.5 to 10.5 min and in relatively similar maximum absorption that demonstrate similar nature of compounds in the tested extracts. Analysis of UV spectra revealed the absorption maxima between 270-300 nm suggesting the phenolic origin of compounds.

4.10. Phytochemical analysis of clove oil

Major ingredient of clove oil as revealed by GC–MS analysis (Table 47) is eugenol (74.32%), and other constituents identified were α-caryophyllene (4.05%), iso-caryophyllene (5.96%), caryophyllene oxide (2.41%), β-caryophyllene (4.92%), napthalene, 1,2,3,5,6,8a-hexahydro- 4,7-dimethyl-1-(1-methyl ethyl) (7.04%) and 1,6-Octadiene- ol-,3,7-dimethyl acetate (1.28%) (table 47 and figure 62).

4.11. Phytochemical analysis of peppermint oil

Major ingredients of peppermint oil as revealed by GC– MS analysis are menthol (36.87%), and other constituents identified were menthone (16.44%), neoisomenthol (11.33%), isomenthone (10.47%), menthyl acetate (7.47%), 2-isopropyl-5-methylcyclohexanol (2.74%), piperitone (2.17%) and limonene (0.53%) as given in table 48 and figure 63.

Tables and figures

Table 1a: Biochemical characteristics of bacteria isolated from clinical, hospital wastewater and environmental sources

Isolates	Gram staining	Indole production	MR test	VP test	Citrate utilization	Starch utilization	Nitrate utilization	Gelatin hydrolysis	Oxidase	H_2S	Catalase	urease	Glucose	fructose	Maltose	Sucrose	Mannitol	Lactose
PAF1	- short rods	-	-	-	+	-	+	+	+	-	+	-	+	-	-	-	+	-
PAF2	-do-	-	-	-	+	-	+	+	+	-	+	-	+	-	-	-	+	-
PAF3	-do-	-	-	-	+	-	+	+	+	-	+	-	+	-	-	-	+	-
PAF4	-do-	-	-	-	+	-	+	+	+	-	+	-	+	-	-	-	+	-
PAF5	-do-	-	-	-	+	-	+	+	+	-	+	-	+	-	-	-	+	-
PAF6	-do-	-	-	-	+	-	+	+	+	-	+	-	+	-	-	-	+	-
PAF7	-do-	-	-	-	+	-	+	+	+	-	+	-	+	-	-	-	+	-
PAF8	-do-	-	-	-	+	-	+	+	+	-	+	-	+	-	-	-	+	-
PAF9	-do-	-	-	-	-	-	+	+	+	-	+	-	+	-	-	-	+	-
PAF10	-do-	-	-	-	+	+	-	+	+	-	+	-	-	-	+	+	+	-
PAF11	-do-	-	-	-	+	-	+	+	+	-	+	-	+	+	+	-	+	-
PAF12	-do-	-	-	-	+	-	+	+	+	-	+	-	-	+	-	-	-	-
PAF13	-do-	-	-	-	+	+	-	+	+	-	+	-	+	-	-	+	+	-
PAF14	-do-	-	-	-	+	-	+	+	+	-	+	-	-	-	-	+	-	-
PAF15	-do-	-	-	-	+	-	+	+	+	-	+	-	+	-	-	-	+	-
PAF16	-do-	-	-	-	+	-	+	+	+	-	+	-	+	-	-	-	+	-
PAF17	-do-	-	-	-	+	-	+	+	+	-	+	-	+	-	-	-	+	-
PAF18	-do-	-	-	-	+	-	+	+	+	-	+	-	+	-	-	-	+	-
PAF19	-do-	-	-	-	+	-	+	+	+	-	+	-	+	-	-	-	+	-
PAF20	-do-	-	-	-	+	-	+	+	+	-	+	-	+	-	-	-	+	-
PAF21	-do-	-	-	-	+	-	+	+	+	-	+	-	+	-	-	-	+	-
PAF22	-do-	-	-	-	+	-	+	+	+	-	+	-	+	-	-	-	+	-
PAF23	-do-	-	-	-	+	-	+	+	+	-	+	-	+	-	-	-	+	-
PAF24	-do-	-	-	-	+	-	+	+	+	-	+	-	+	-	-	-	+	-
PAF25	-do-	-	-	-	+	-	+	+	+	-	+	-	+	-	-	-	+	-
PAF26	-do-	-	-	-	+	-	+	+	+	-	+	-	+	-	-	-	+	-
PAF27	-do-	-	-	-	+	+	-	+	+	-	+	-	+	-	-	-	+	-
PAF28	-do-	-	-	-	+	-	-	-	+	-	+	-	-	-	-	+	+	-
PAF29	-do-	-	+	-	+	-	+	-	+	-	+	-	+	-	-	-	-	-
PAF30	-do-	-	-	-	+	-	+	+	+	-	+	-	-	+	+	-	-	-
PAF31	-do-	-	-	-	+	-	-	+	+	-	+	-	-	+	-	-	+	-
PAF32	-do-	-	-	-	+	+	-	+	+	-	+	-	+	+	+	+	+	-
PAF33	-do-	-	-	-	+	+	+	-	+	-	+	-	-	-	+	+	-	-
PAF34	-do-	-	-	-	+	-	+	+	+	-	+	-	+	-	-	-	+	-
PAF35	-do-	+	-	-	+	+	-	-	+	-	+	-	-	+	-	-	+	-
PAF51	-do-	-	-	-	+	-	+	+	+	-	+	-	+	-	-	-	-	-
PAF52	-do-	-	-	-	+	-	+	+	+	-	+	-	-	-	-	-	+	-
PAF53	-do-	-	-	-	+	-	+	+	+	-	+	-	+	-	-	-	+	-

PAF54	-do-	-	-	-	+	-	+	+	+	-	+	-	+	-	-	-	+	-
PAF79	-do-	-	-	-	+	-	+	+	+	-	+	-	+	-	-	-	+	-
WAF36	-do-	-	-	+	+	-	+	-	-	-	+	+	+	+	+	+	+	+
WAF37	-do-	-	-	+	+	-	+	-	-	-	+	+	+	+	+	+	+	+
WAF38	-do-	+	+	+	-	-	+	+	+	+	+	-	+	+	+	+	+	-
WAF39	-do-	-	-	-	+	-	+	-	-	-	-	-	+	-	+	+	+	+
WAF40	-do-	-	+	-	+	-	+	-	-	-	-	+	+	-	+	+	+	+
WAF41	-do-	-	-	-	+	-	+	-	-	-	+	-	+	+	+	+	+	-
WAF42	-do-	-	+	+	+	-	+	-	-	-	+	-	+	-	+	+	+	+
WAF43	-do-	-	-	-	+	-	-	+	+	-	+	-	+	+	+	+	+	+
WAF44	-do-	-	-	-	+	+	-	+	+	-	+	-	-	-	+	+	-	-
WAF45	-do-	-	-	-	+	+	+	-	+	-	+	-	+	-	-	-	+	-
WAF46	-do-	-	-	-	+	-	+	+	+	-	-	+	-	+	-	-	+	-
WAF47	-do-	+	+	+	-	-	+	+	+	+	+	-	+	+	+	+	+	+
WAF48	-do-	-	-	-	+	-	-	+	+	-	+	-	+	+	+	+	+	-
WAF49	-do-	-	-	-	+	+	-	+	+	-	+	-	-	-	+	+	-	-
WAF50	-do-	-	-	-	+	+	+	-	+	-	+	-	+	-	-	-	+	-

+ indicates positive test; - indicates negative test

Table 1b: Bacterial isolates and their source

Bacteria identified	Source	Total number	Isolate designation
Pseudomonas aeruginosa	Clinical	26	PAF1-PAF8, PAF14-PAF26, PAF51-PAF54, PAF79
Pseudomonas sp	Soil contaminated witrh hospital wastewater	14	PAF9-PAF13, PAF27-PAF35,
Klebsiella sp	Hospital wastewater	06	WAF36-WAF37 WAF39-WAF42
Pseudomonas sp	Hospital wastewater	07	WAF43-46, WAF48-50
Aeromonas sp	Hospital wastewater	02	WAF38, WAF47

Table 2: Antibiotic sensitivity profile of isolated bacteria by disc diffusion method

Isolates	Antibiotics (Zone of inhibition diameter in mm)																
	Ax	Ao	Cfp	Cxm	Cx	Ctr	Cip	Cd	Do	E	Gf	G	I	K	Nf	Pb	Sc
PAF1	R	R	R	R	15	20	16	R	14	R	17	R	26	R	R	R	20
PAF2	R	R	R	R	18	16	22	R	18	R	R	16	22	18	R	20	R
PAF3	21	R	16	19	16	20	19	21	16	23	20	21	27	16	20	17	23
PAF4	R	R	R	R	17	19	21	R	21	R	15	21	24	23	R	15	29
PAF5	R	24	R	R	20	25	18	17	18	25	15	18	21	17	R	R	24
PAF6	22	R	23	15	16	18	22	18	26	R	16	22	28	18	15	18	R
PAF7	R	R	R	15	18	24	R	17	22	25	19	R	21	R	21	R	19
PAF8	R	R	R	R	15	19	20	20	17	R	22	15	25	21	R	21	R
PAF9	R	R	23	R	R	18	25	21	26	24	25	19	30	24	R	R	33
PAF10	R	R	29	R	R	23	29	21	28	R	21	25	25	22	R	24	24
PAF11	17	22	32	20	18	28	28	22	22	R	24	28	28	21	19	19	R
PAF12	R	R	20	R	18	17	24	18	19	26	20	26	24	25	23	25	R
PAF13	R	R	25	20	18	25	28	25	18	22	27	22	23	18	22	R	26
PAF14	R	R	19	25	R	23	17	16	25	19	16	24	R	22	25	R	22
PAF15	R	R	17	18	17	26	18	22	21	26	21	R	25	24	16	16	27
PAF16	18	R	23	16	16	22	21	R	23	21	24	21	22	R	20	19	R
PAF17	23	R	20	21	21	25	24	20	R	24	19	17	27	23	19	23	21
PAF18	18	R	24	R	20	20	27	R	15	25	22	17	25	20	R	21	24
PAF19	22	22	25	R	15	26	23	R	14	18	18	19	28	25	R	R	26
PAF20	21	R	17	25	18	19	24	17	R	20	20	18	24	18	R	15	29
PAF21	20	25	17	18	19	R	22	15	21	16	24	21	22	26	17	19	19
PAF22	19	R	21	R	19	27	24	R	19	21	19	20	23	21	R	21	23
PAF23	R	R	25	17	23	17	24	R	R	R	19	R	26	R	R	24	26
PAF24	19	R	18	R	17	25	18	R	22	24	26	16	20	17	R	16	22
PAF25	R	R	27	R	17	18	26	R	25	20	23	20	24	22	24	22	27
PAF26	17	21	20	21	R	16	21	15	R	R	18	24	19	24	R	R	20
PAF27	R	R	28	R	24	R	21	R	27	18	16	25	26	R	R	R	22
PAF28	R	R	22	17	21	22	25	20	22	25	21	21	22	22	25	R	30
PAF29	27	R	24	17	20	23	19	R	R	R	24	27	19	24	25	R	27
PAF30	R	18	21	21	R	17	24	R	R	21	19	24	31	26	R	20	24
PAF31	20	22	21	22	15	19	21	R	25	R	27	22	26	29	20	R	22

PAF32	20	21	17	16	19	24	22	R	R	R	16	21	29	23	26	R	28
PAF33	R	25	16	R	27	20	20	R	21	R	26	19	26	22	21	R	R
PAF34	19	R	27	18	20	20	23	21	R	R	22	23	28	25	19	R	25
PAF35	R	24	30	23	R	21	18	25	24	24	28	18	R	23	24	16	20
PAF 51	R	19	22	18	19	R	R	18	20	R	R	18	24	R	20	R	18
PAF52	18	26	19	27	18	21	27	18	20	R	15	21	20	25	16	18	24
PAF53	R	22	24	20	R	R	19	23	22	R	22	R	27	19	R	21	19
PAF54	15	20	21	15	22	R	R	19	21	R	R	19	27	R	20	R	25
PAF79	R	R	28	R	R	16	20	R	23	R	23	R	23	R	R	R	28
WAF36	23	27	29	21	R	26	23	R	R	18	23	28	20	17	17	R	24
WAF37	24	21	20	28	17	21	26	22	18	23	26	21	R	20	16	R	R
WAF38	R	R	24	25	R	17	21	15	R	20	20	23	25	25	25	R	R
WAF39	19	R	26	22	23	24	21	26	21	R	19	26	22	24	R	22	26
WAF40	16	R	19	23	21	R	27	21	26	R	27	15	27	25	21	17	21
WAF41	21	R	17	20	16	R	R	24	29	21	22	R	23	18	24	20	19
WAF42	18	33	28	R	24	19	25	22	25	23	21	19	28	R	20	20	23
WAF43	23	19	16	18	21	16	18	26	R	17	19	25	20	R	17	16	28
WAF44	R	R	21	16	26	22	22	29	19	R	17	26	25	R	25	19	25
WAF45	25	R	22	R	21	R	25	16	R	R	22	27	29	R	15	22	20
WAF46	16	21	22	21	18	R	20	R	22	R	29	21	18	R	R	18	23
WAF47	R	22	25	15	R	R	24	R	24	20	16	25	R	R	23	15	25
WAF48	18	31	18	29	22	R	24	22	27	18	21	26	23	R	20	19	23
WAF49	18	R	19	24	16	R	20	23	R	R	24	23	32	24	R	24	31
WAF 50	R	26	23	19	21	R	R	26	R	R	18	20	19	20	23	21	25

Ax- Amoxicillin, Ao- Aztreonam, Cfp- Cefpirome, Cxm- Cefuroxime, Cx- Cephoxitin, Ctx- Ceftriaxone, Cip- Ciprofloxacin, Cd- Clindamycin, Do- Doxycycline , E- Erythromycin, Gf- Gatifloxacin, G-Gentamycin, I- Imepenem, K- Kanamycin, Nf- Nitrofurontoin, Ox- Oxacillin, Sc-Sparfloxacin, R- Resistant

Table 3: Incidence of antibiotic resistance among bacterial isolates

Antibiotics	Potency (µg/disc)	Isolate designation	Percent resistant isolates (%)
Amoxicillin (Ax)	10	PAF1-2, PAF4-5, PAF7-10, PAF12-15, PAF23, PAF25, PAF27-28, PAF30, PAF33, PAF35, WAF38, WAF44, WAF47, WAF50, PAF51, PAF53, PAF79	47.2
Aztreonam (Ao)	10	PAF1-4, PAF6-10, PAF12-18, PAF20, PAF22-25, PAF27-29, PAF34, WAF38, WAF39-41, WAF44-45, WAF49, PAF79	60
Cefpirome (Cfp)	30	PAF1-2, PAF4-5 PAF7-8	10.9
Cefuroxime (Cxm)	30	PAF1-2, PAF4-5, PAF7-10, PAF12, PAF18-19, PAF22, PAF24-25, PAF27, PAF33, WAF42, WAF45, PAF79	32.7
Cephoxitin (Cx)	30	PAF9-10, PAF14, PAF26, PAF30, PAF35, WAF36, WAF37, WAF47, PAF53, PAF79	20
Ceftriaxone (Ctr)	30	PAF21, PAF27, WAF40-41, WAF45-50, PAF51, PAF53-54	23.6
Ciprofloxacin (Cip)	30	PAF7, WAF41, WAF50, PAF51, PAF54	09.09
Clindamycin (Cd)	30	PAF1-2, PAF4, PAF16, PAF18-19, PAF22-25, PAF27, PAF29-33, WAF36, WAF46-47, PAF79	36.3
Doxycycline (Do)	30	PAF17, PAF20, PAF23, PAF26, PAF29-30, PAF32, PAF34, WAF36, WAF38, WAF43, WAF45, WAF49-50	25.4
Erythromycin (E)	30	PAF1-2, PAF4, PAF6, PAF8, PAF10-11, PAF23, PAF26, PAF29, PAF31-34, WAF39-40, WAF44-46, WAF49-50, PAF51-54, PAF79	47.2
Gatifloxacin (Gf)	30	PAF2, PAF51, PAF54	05.40
Gentamicin (G)	15	PAF1, PAF7, PAF15, PAF23, WAF41, PAF53, PAF79	12.7
Imepenem (I)	10	PAF14, PAF35, WAF37, WAF47	07.20
Kanamycin (K)	30	PAF1, PAF7, PAF16, PAF23, PAF27, WAF42-48, PAF51, PAF54, PAF79	27.2
Nitrofurontoin (Nf)	30	PAF1-2, PAF4-5, PAF8, PAF9-10, PAF18-20, PAF22-24, PAF26, PAF27, PAF30, WAF39, WAF46, WAF49, PAF53, PAF79	38.1
Polymixin-B (Pb)	100 units	PAF1, PAF5, PAF7, PAF9, PAF13, PAF14, PAF19, PAF26, PAF27-29, PAF31-34, WAF36-38, PAF51, PAF54, PAF79	38.1
Sparfloxacin (Sc)	10	PAF2, PAF6, PAF8, PAF11-12, PAF16, PAF33, WAF37-38	16.3

Table 4: Antibiotic resistance pattern among the bacterial isolates

No. of antibiotics	Resistance pattern	Isolate designation
1	Ao	PAF3
	Ctr	PAF21
	E	PAF52, PAF11
2	Ao, Do	PAF17
	Ctr, K	WAF48
	Cxm, K	WAF42
	Do, K	WAF43
3	Ax, Ao, G	PAF15
	Ao, E, Sc	PAF6
	Ao, Do, Nf	PAF20
	Ax, Ao, Pb	PAF13, PAF28
	Cd, E, Pb	PAF31
	Ax, Cx, I	PAF35
	I, Pb, Sc	WAF37
	Ao, E, Nf	WAF39
	Ao, Ctr, E	WAF40
4	Ao, Cd, K, Sc	PAF16
	Ao, Cxm, Cd, Nf	PAF18, PAF24
	Cxm, Cd, Nf, Pb	PAF19
	Ax, Ao, Cxm, Cd	PAF25
	Ax, Ao, Cxm, Sc	PAF12
	Cd, Do, E, Pb	PAF32
	Ao, Do, E, Pb	PAF34
	Cx, Cd, Do, Pb	WAF36
	Ao, Ctr, Cip, G	WAF41
	Ax, Ao, E, K	WAF44
5	Ax, Cfp, Cxm, Nf, Pb	PAF5
	Ax, Ao, Cx, I, Pb	PAF14
	Cx, Do, E, Nf, Pb	PAF26
	Ao, Cd, Do, E, Pb	PAF29
	Ao, Cx, Cd, Do, Nf	PAF30
	Ao, Ctr, Do, E, Nf	WAF49
	Ax, Ctr, Cip, Do, E	WAF50
	Ctr, Cd, E, K, Nf	WAF46
6	Ax, Cx, Ctr, E, G, Nf	PAF53
	Ctr, Cip, E, Gf, K, Pb	PAF54
	Ax, Ao, Cxm, Cx, Nf, Pb	PAF9
	Ax, Ao, Cxm, Cx, E, Nf	PAF10
	Ax, Cxm, Cd, E, Pb, Sc	PAF33
	Ax, Ao, Cx, Do, Pb, Sc	WAF38
	Ao, Cxm, Ctr, do, E, K	WAF45
	Ax, Cx, Ctr, Cd, I, K	WAF47
7	Ax, Ao, Cfp, Cip, G, K, Pb	PAF7
	Ax, Ao, Cfp, Cxm, E, Nf, Sc	PAF8
	Ax, Ao, Cd, Do, E, G, K, Nf	PAF23
	Ax, Ctr, Cip, E, Gf, K, Pb	PAF51
	Ax, Ao, Cfp, Cxm, Cd, E, Nf	PAF4
	Ax, Ao, Cxm, Ctr, Cd, K, Nf, Pb	PAF27
9	Ax, Ao, Cfp, Cxm, Cd, E, Gf, Nf, Sc	PAF2
10	Ax, Ao, Cfp, Cxm, Cd, E, G, K, Nf, Pb	PAF1
	Ax, Ao, Cxm, Cx, Cd, E, G, K, Nf, Pb	PAF79

Table 5: Screening of bacterial isolates for AHL production using biosensor strains

Isolate designation	AHL production		Percent of isolates
	CVO26	A136	
PAF1, PAF2, PAF4, PAF6, PAF7, PAF8, PAF9, PAF10, PAF14, PAF16, PAF17, PAF19, PAF21, PAF23, PAF24, PAF26, PAF27, PAF32, PAF33, PAF35, WAF38, WAF43, WAF44, WAF47, WAF49, WAF50, PAF51, PAF54, PAF-79	+	+	52.7
PAF3, PAF5, PAF11, PAF12, PAF13, PAF15, PAF18, PAF20, PAF22, PAF25, PAF28-31, PAF34, WAF37, WAF39-42, WAF45-46, WAF48, PAF52-53	ND	ND	47.3

+, AHL detected; ND- AHL not detected

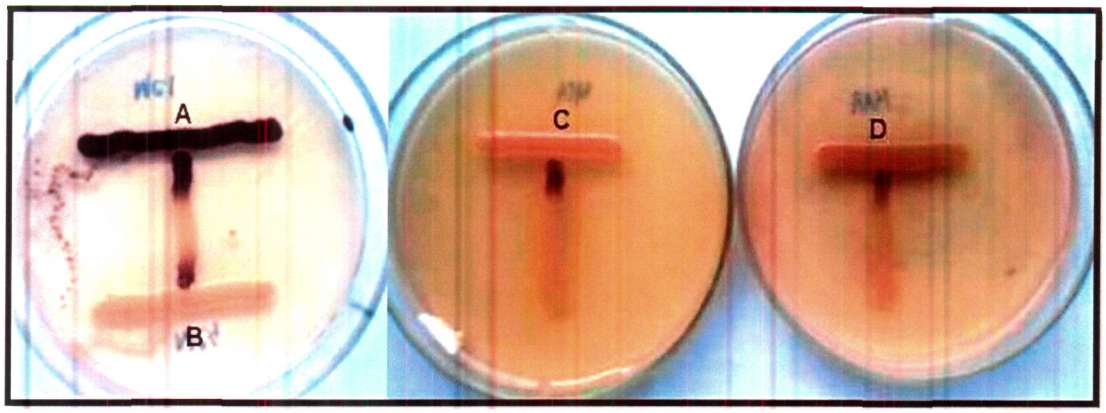


Plate 1: T-streak assay for screening for production of acylated homoserine lactone (AHL) in isolated bacteria using *C. violaceum* CV026. A). *Chromobacterium violaceum* 12472 (positive control); B). *Pseudomonas aeruginosa* PAO1 (postive control); C). WAF38; D). PAF79

Plate 2: Agar plate assays for screening for production of acylated homoserine lactone (AHL) in isolated bacteria using *A. tumefaciens* A136. 1) Screening of bacteria for AHL production; 1a. No AHL production; 1b , 1c. isolates postive for AHL production. 2). AHL production by 2a. PAF14 and 2b. PAF79; 3). AHL production by 3a. WAF38 and 3b. WAF47; 4a. Postive control, 4b. Negative control

Table 6: Analyses of AHLs in the extracts of spent culture supernatants from AHL producing bacteria

Bacterial strains	AHLs detected by TLC bioautography	Mass spectra
Pseudomonas aeruginosa PAF1	C4-HSL	
Pseudomonas aeruginosa PAF2	C4-HSL, C6-HSL, 3-oxo-C12 HSL, unidentified	
Pseudomonas aeruginosa PAF4	C4-HSL	
Pseudomonas aeruginosa PAF6	C4-HSL, 3-oxo-C12 HSL	
Pseudomonas aeruginosa PAF7	C4-HSL	
Pseudomonas aeruginosa PAF8	C4-HSL, C6-HSL, 3-oxo-C12 HSL	
Pseudomonas sp.PAF9	C4-HSL	
Pseudomonas sp. PAF10	C4-HSL	
Pseudomonas aeruginosa PAF14	C4-HSL, C6-HSL, unidentified	C4-HSL, C6-HSL
Pseudomonas aeruginosa PAF16	C4-HSL	
Pseudomonas aeruginosa PAF17	C4-HSL	
Pseudomonas aeruginosa PAF19	C4-HSL, C6-HSL, 3-oxo-C12 HSL	
Pseudomonas aeruginosa PAF21	C4-HSL	
Pseudomonas aeruginosa PAF23	C4-HSL, C6-HSL	
Pseudomonas aeruginosa PAF24	C4-HSL	
Pseudomonas aeruginosa PAF26	C4-HSL, C6-HSL, 3-oxo-C12 HSL, unidentified	
Pseudomonas sp. PAF27	C4-HSL	
Pseudomonas sp. PAF32	C4- HSL	
Pseudomonas aeruginosa PAF33	C4- HSL, 3-oxo-C12 HSL	
Pseudomonas sp PAF35	C4- HSL	
Pseudomonas aeruginosa PAF51	C4- HSL, 3-oxo-C12 HSL	
Pseudomonas aeruginosa PAF54	C4- HSL	
Pseudomonas aeruginosa PAF79	C4-HSL, C6-HSL, 3-oxo-C12 HSL, unidentified	C4-HSL, C6-HSL, 3-oxo-C12 HSL
Aermonas hydrophila WAF38	C4-HSL, C6-HSL	C4-HSL, C6-HSL
Pseudomonas sp. WAF43	C4-HSL	
Pseudomonas sp. WAF44	C4-HSL, C6-HSL	
Aermonas aquariorum WAF47	C4-HSL, C6-HSL, 3-oxo-C12 HSL, unidentified	C4-HSL, C6-HSL, 3-oxo-C12 HSL
Pseudomonas sp.WAF49	C4-HSL	
Pseudomonas aeruginosa WAF50	C4-HSL, 3-oxo-C12 HSL	

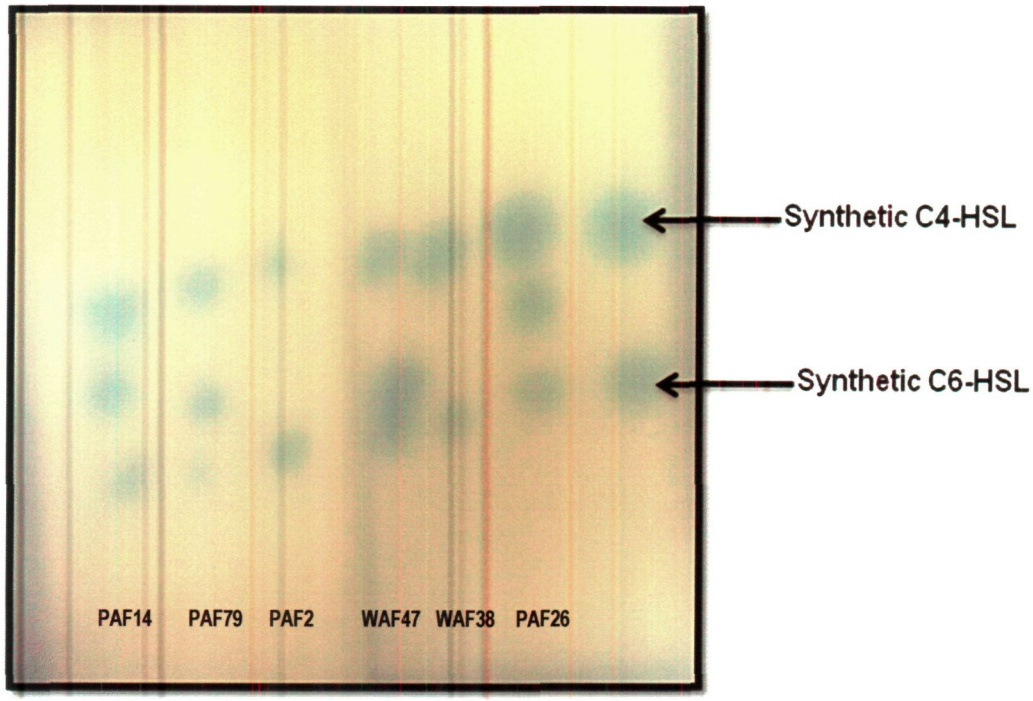


Plate 3: Separation and detection of acyl- HSL standards and acyl- HSLs produced by isolates using TLC with *Chromobacterium violaceum* CVO26 strain

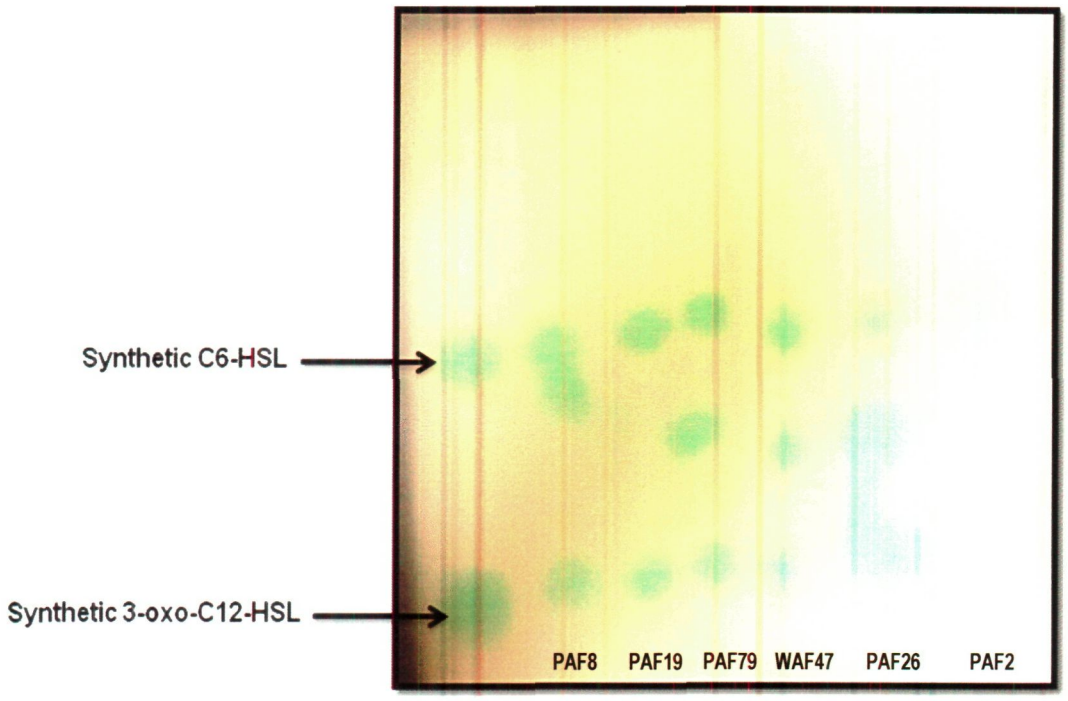


Plate 4: Separation and detection of acyl- HSL standards and acyl- HSLs produced by isolates using TLC with *Agrobacterium tumefaciens* A136 strain.

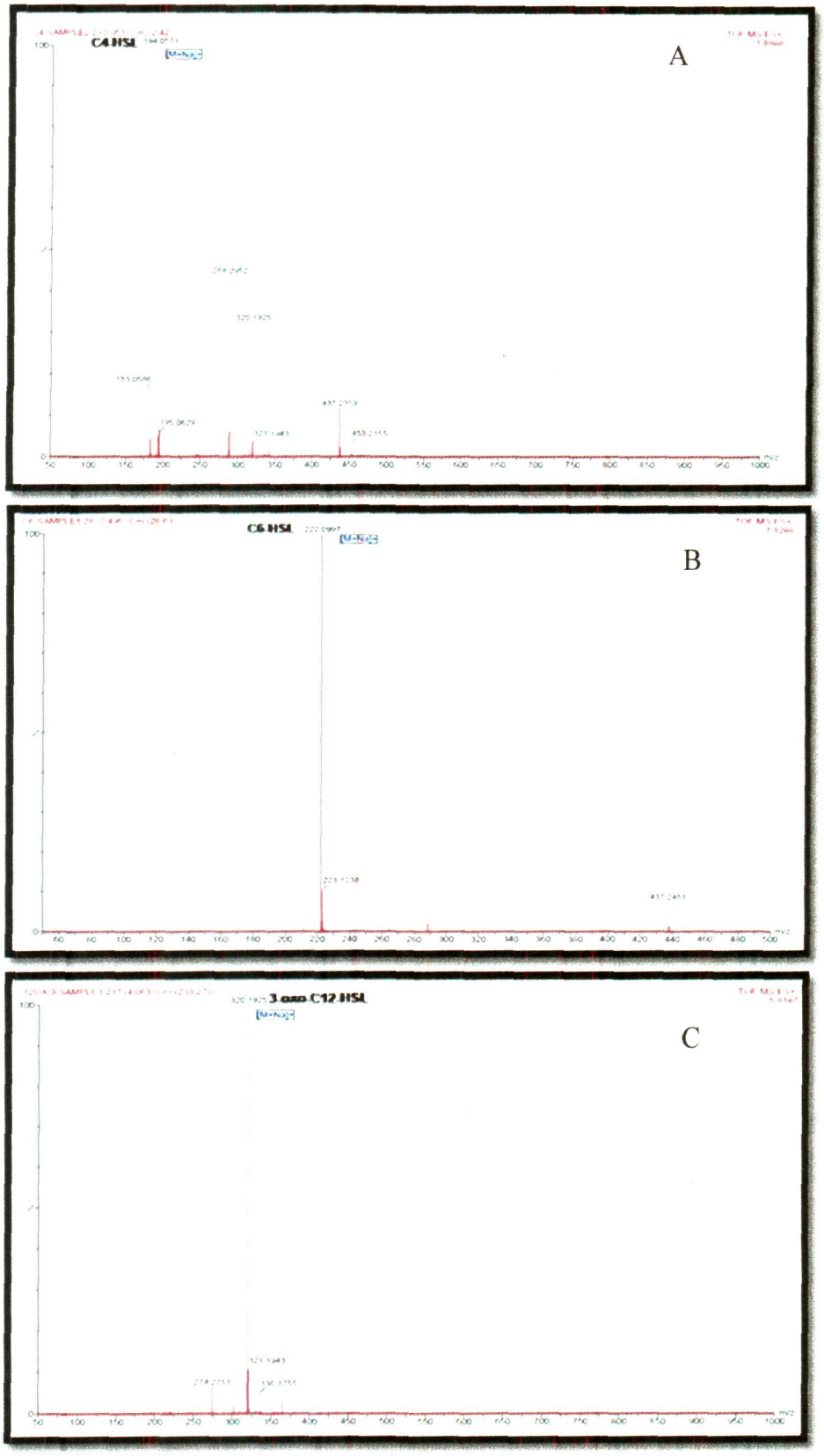


Figure1: MS analyses of standard synthetic AHLs (**A**) Mass spectra of C4-HSL (**B**) Mass spectra of C6-HSL (**C**). Mass spectra of 3-oxo- C12-HSL.

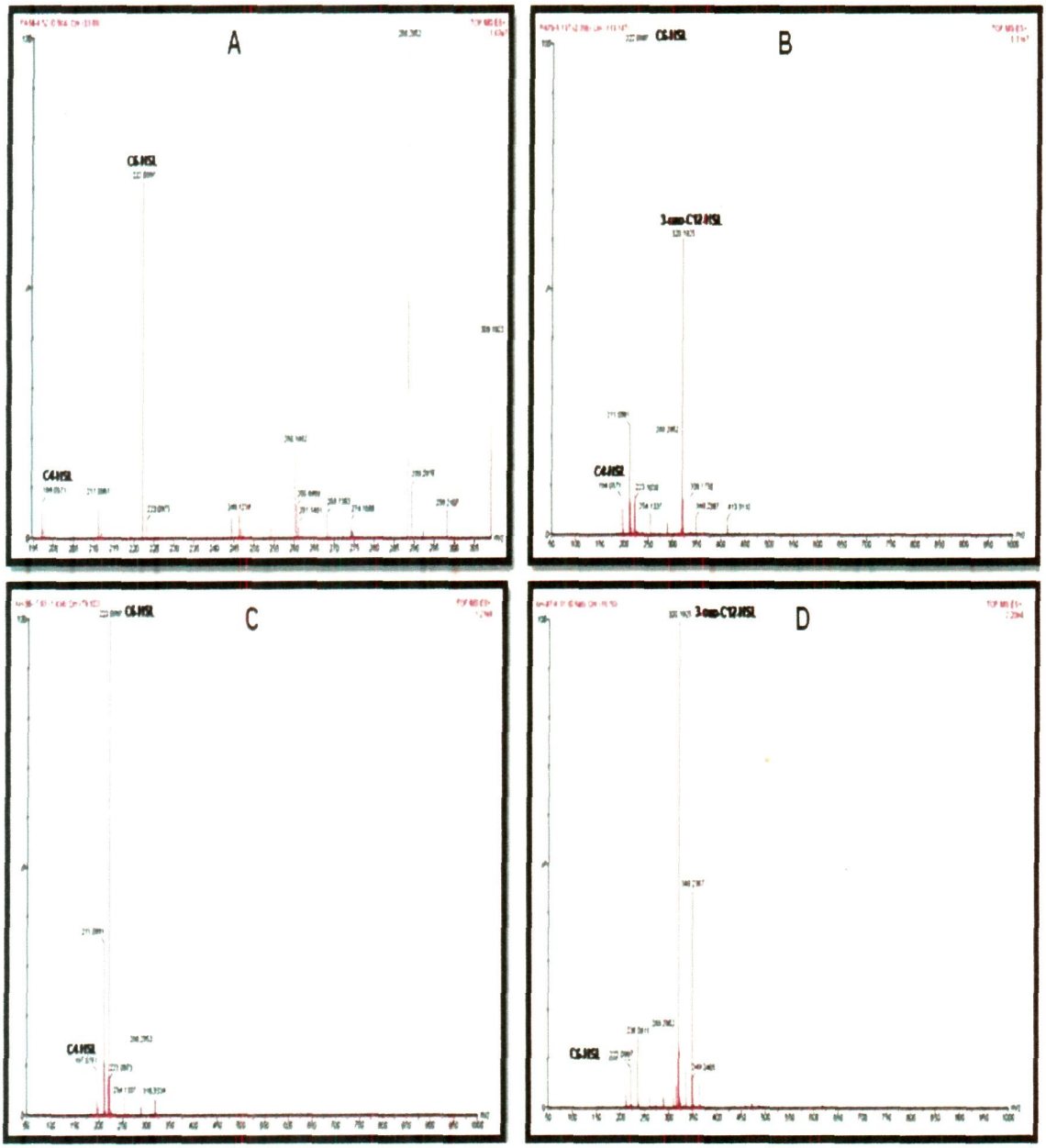


Figure 2: MS analyses of the extract of spent culture supernatant from isolated strains. A). PAF14 B). PAF79 C). WAF38 D). WAF47

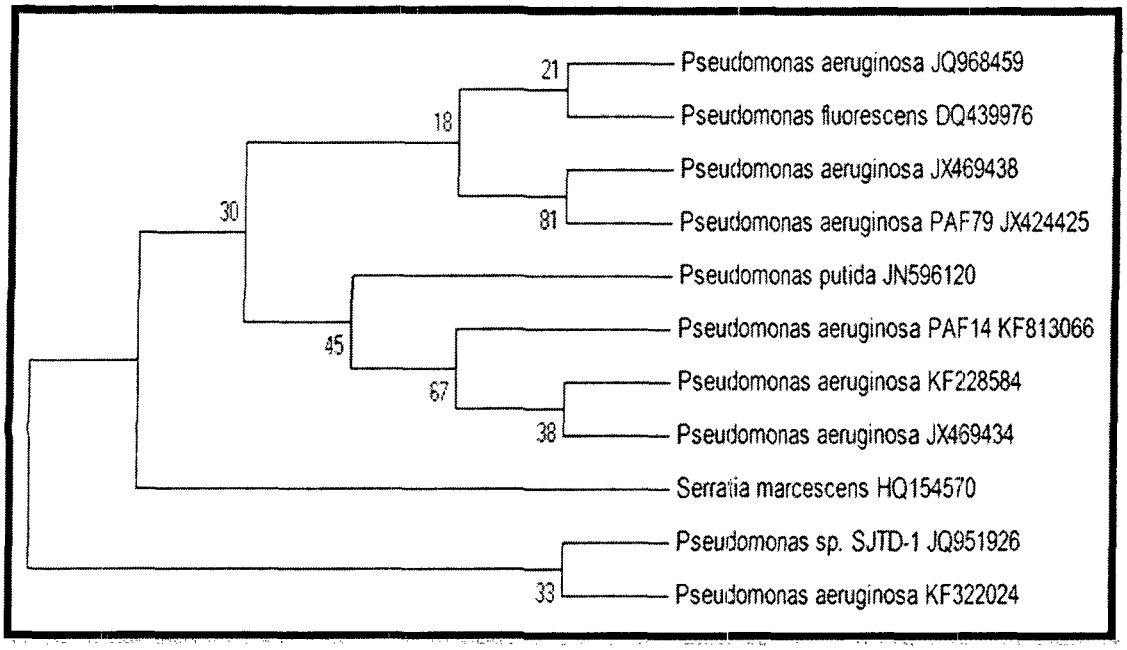


Figure 3: 16S rDNA gene sequence based phylogenetic tree showing the phylogenetic positions of isolated bacterial isolates PAF14 and PAF79.

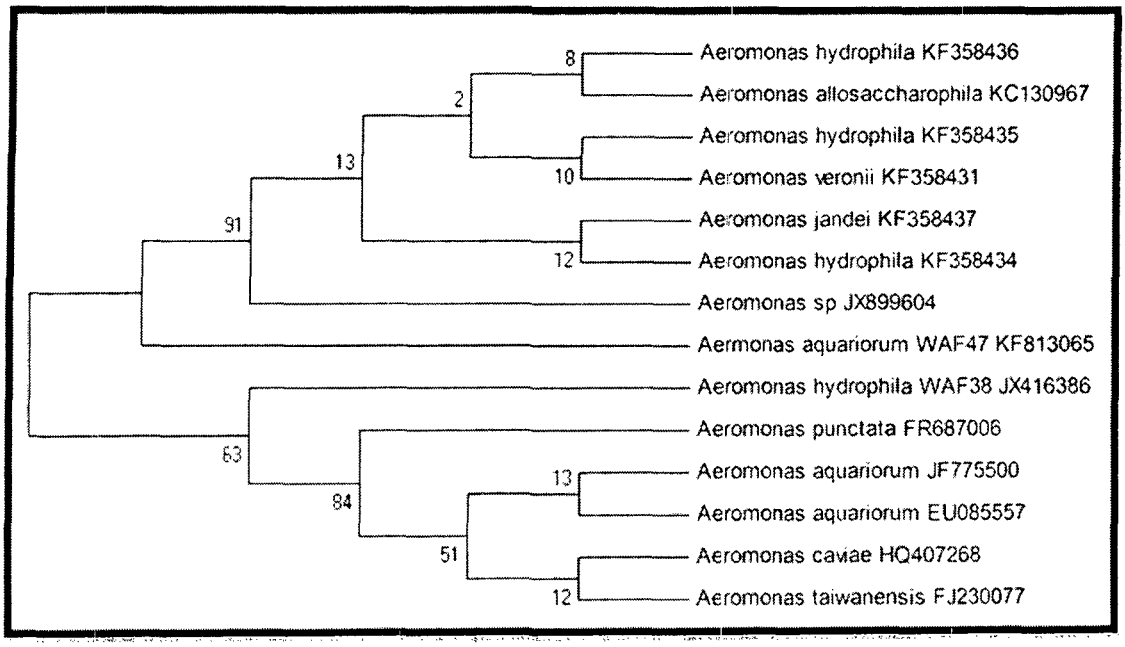


Figure 4: 16S rDNA gene sequence based phylogenetic tree showing the phylogenetic positions of isolated bacterial isolates WAF38 and WAF47.

Table 7: Detection of quorum sensing mediated virulence factors in AHL producing isolates

Isolates	Virulence factors						
	Elastase[a]	Total protease[b]	Pyocyanin production[c]	Chitinase[d]	EPS production[e]	Swarming motility[f]	Biofilm formation[g]
PAO1	0.148±0.018	1.124±0.032	5.8±0.6	0.134±0.013	1.01±0.035	72±2.3	0.457±0.022
PAF1	0.053±0.014	0.342±0.021	5.1±0.9	0.041±0.007	0.993±0.024	68±3.9	0.215±0.026
PAF2	0.131±0.021	0.881±0.035	6±1.2	0.117±0.024	1.1±0.033	55±1.8	0.254±0.017
PAF4	0.110±0.016	0.332±0.018	4.7±0.7	0.094±0.019	0.618±0.028	33±1.4	0.094±0.009
PAF6	0.076±0.021	0.553±0.026	5.1±1.3	0.067±0.023	0.615±0.019	42±0.8	0.164±0.016
PAF7	0.044±0.006	0.221±0.018	3.6±0.8	0.078±0.005	0.229±0.021	45±1	0.336±0.024
PAF8	0.118±0.018	0.752±0.035	4.6±1.9	0.1±0.012	0.645±0.029	38±0.5	0.388±0.018
PAF9	0.024±0.011	0.622±0.026	1.5±0.4	0.029±0.017	0.449±0.022	13±2.4	0.315±0.021
PAF10	0.017±0.01	0.405±0.017	1.9±0.2	0.022±0.004	0.223±0.028	19±1.6	0.228±0.03
PAF14	0.121±0.021	0.914±0.034	4.9±2.7	0.142±0.018	0.976±0.041	14±0.4	0.357±0.025
PAF16	0.039±0.008	0.254±0.02	2.9±0.4	0.099±0.014	0.765±0.028	41±0.7	0.162±0.012
PAF17	0.054±0.01	0.217±0.013	3.6±2.1	0.058±0.013	0.322±0.021	33±1.1	0.094±0.02
PAF19	0.077±0.016	0.542±0.021	4.4±1.5	0.034±0.005	0.219±0.027	40±1.7	0.123±0.018
PAF21	0.048±0.013	0.199±0.018	3.6±1.9	0.037±0.005	0.275±0.016	22±0.5	0.099±0.015
PAF23	0.099±0.015	0.549±0.033	4.8±2.8	0.091±0.022	0.812±0.037	41±3.2	0.301±0.023
PAF24	0.061±0.012	0.414±0.024	4.1±0.6	0.091±0.025	0.787±0.029	55±3.8	0.339±0.014
PAF26	0.079±0.016	0.447±0.026	3.2±0.9	0.054±0.018	0.615±0.021	49±1.5	0.276±0.02
PAF27	0.021±0.001	0.331±0.022	0.8±0.02	0.038±0.008	0.542±0.026	10±0.2	0.181±0.016
PAF32	0.027±0.004	0.226±0.012	1.4±0.01	0.048±0.01	0.212±0.018	10±0.1	0.225±0.023
PAF33	0.015±0.002	0.288±0.021	2±0.9	0.061±0.011	0.440±0.022	14±0.3	0.101±0.018
PAF35	0.033±0.008	0.154±0.017	0.6±0.01	0.030±0.009	0.256±0.019	17±0.5	0.220±0.015
PAF51	0.076±0.012	0.812±0.028	4.4±0.5	0.089±0.014	0.648±0.027	56±2.5	0.419±0.024
PAF54	0.044±0.006	0.336±0.018	4.7±0.9	0.097±0.009	0.521±0.021	46±1.7	0.401±0.027
PAF79*	0.151±0.018	1.01±0.032	5±1.2	0.123±0.01	0.992±0.024	59±0.7	0.393±0.022
WAF43	0.053±0.004	0.243±0.017	1.7±0.7	0.032±0.019	0.324±0.021	18±0.6	0.251±0.016
WAF44	0.046±0.01	0.169±0.023	0.9±0.06	0.074±0.014	0.220±0.016	13±0.4	0.184±0.02
WAF48	0.048±0.006	0.248±0.027	0.3±0.03	0.031±0.004	0.263±0.02	22±0.9	0.180±0.017
WAF50	0.028±0.009	0.187±0.017	0.8±0.03	0.036±0.011	0.157±0.017	29±1.2	0.142±0.013
WAF38*	ND	0.582±0.021	ND	ND	0.623±0.028	ND	0.288±0.016
WAF47	ND	0.514±0.019	ND	ND	0.618±0.017	ND	0.269±0.021

[a]Elastase activity is expressed as the absorbance at OD_{495}.
[b]Total protease activity is expressed as the absorbance at OD_{600}.
[c]Pyocyanin concentrations were expressed as micrograms of pyocyanin produced per microgram of total protein.
[d]Chitinase activity is expressed as the absorbance at OD_{570}.
[e] EPS production is expressed as absorbance at OD_{480}
[f]Swarming motility is expressed as diameter of swarm in mm
[g]Biofilm formation is expressed as OD_{470} after incubation with crystal violet
ND- not detected
*- isolates selected for further studies

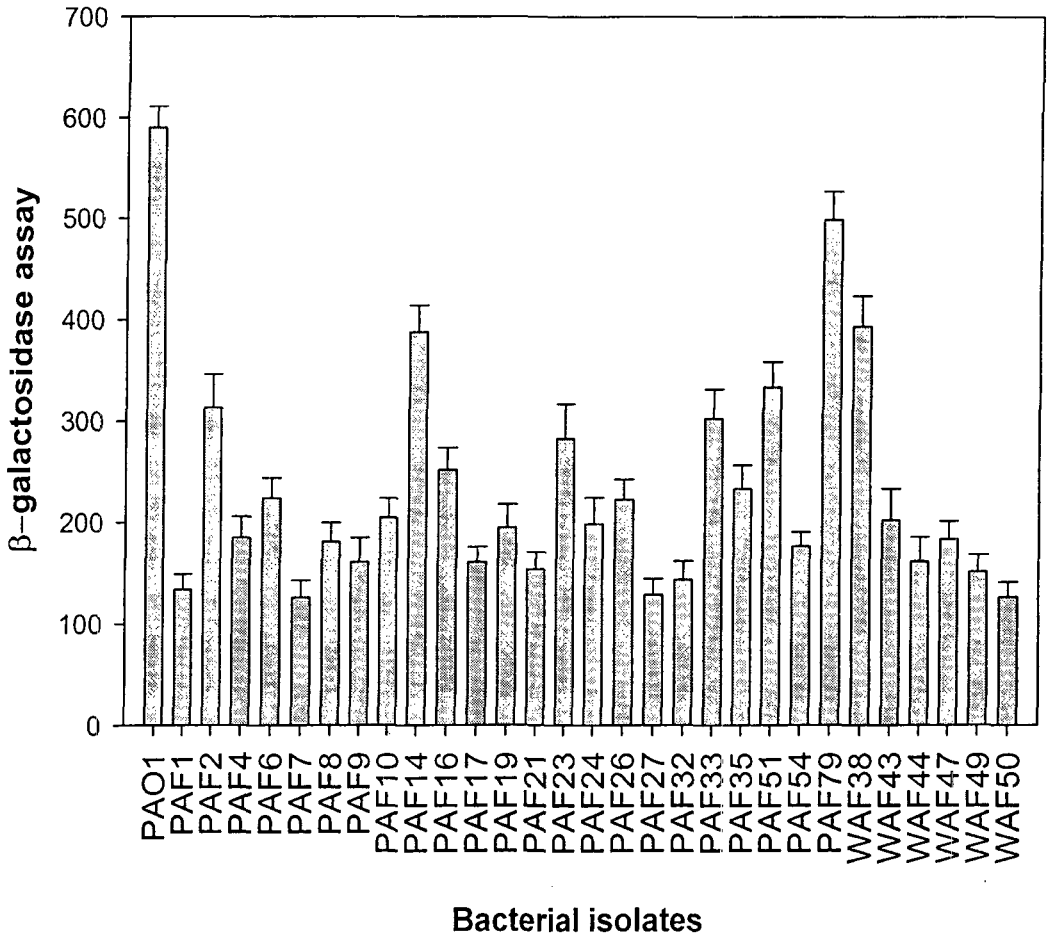


Figure 5: Quantitative β-galactosidase activity expressed in miller units (MU) for determining the levels of AHL molecules in isolated bacteria.

Table 8: Screening of certain common antibiotics for their possible pigment (violacein) inhibition activity

Antibiotics	Disc potency (µg)	Violacein inhibition	
		CV12472	CVO26
Active antibiotics			
Ceftazidime (Caz)[b]	30	17±0.8	14±0.9
Ciprofloxacin (Cip)[a]	5	8±0.6	5±0.4
Doxycycline (Do)[b]	30	16±0.5	18±0.8
Erythromycin (E)[b]	15	8±0.3	5±0.2
Kanamycin (K)[b]	30	4±0.3	4±0.3
Tobramycin (Tb)[a]	10	09±0.2	04±0.5
Inactive antibiotics			
Amoxycillin (A)	10		
Aztreonam (Ao)	30		
Bacitracin (B)	10		
Cefepime (Cpm)	30		
Cefpodoxime (Cpd)	10		
Cefuroxime (Cxm)	30		
Cephotaxime (Ce)	30		
Cephoxitin (Cx)	30	No pigment inhibition activity	
Chloramphenicol (C)	30		
Colistin (Cl)	10		
Gentamicin (G)	10		
Imipenem (I)	10		
Levofloxacin (Le)	5		
Meropenem (Mr)	10		
Methicillin (M)	5		
Nalidixic Acid (Na)	30		
Nitrofurantoin (Nf)	300		
Novobiocin (Nv)	30		
Oxacillin (Ox)	1		
Piperacillin (Pc)	100		
Polymixin B (Pb)	300 units		
Rifampicin (R)	5		
Sparfloxacin (Sc)	5		
Streptomycin (S)	10		
Tetracycline (T)	30		
Vancomycin (Va)	30		

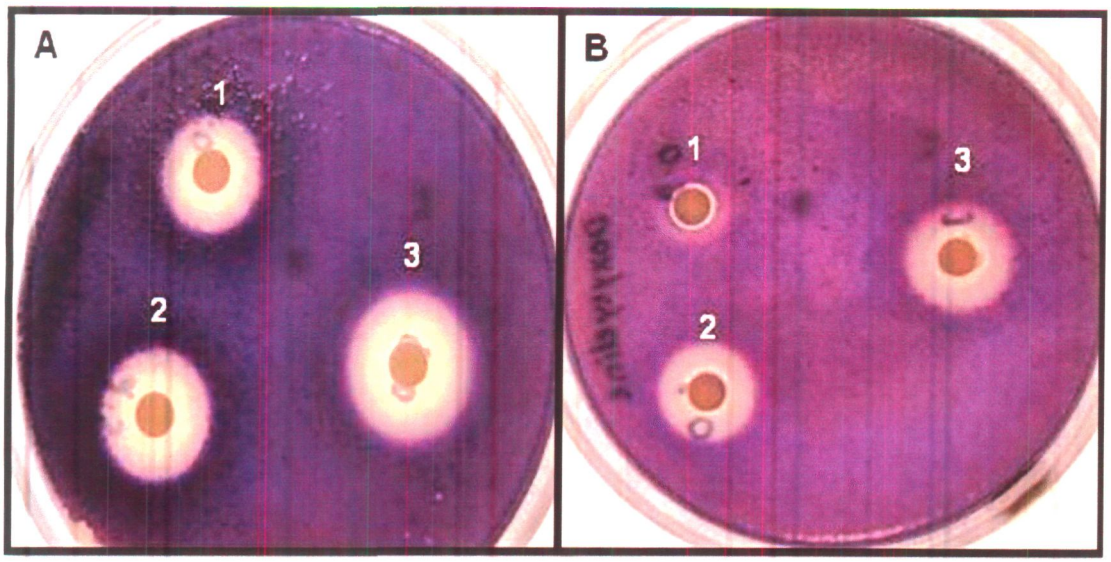


Plate 5: Inhibition of violacein by A). Doxycycline, (1) 1 µg/ml, (2) 1 µg/ml, (3) 4 µg/ml; B). Ceftazidime, (1) 0.06 µg/ml, (2) 0.125 µg/ml, (3) 0.25 µg/ml in *C. violaceum* CV12472

Table 9: Screening of methanolic extracts of Indian medicinal plants for their pigment (violacein) inhibitory activity against *Chromobacterium violaceum* strains

Plant	Common name	Part used	Yield (%)	Interference of violacein production	
				CV12472	CV026
Active extracts					
Camelia sinensis	Tea	Leaves	14.6	15±0.7	7±0.2
Cuminum cyminum	Jira	Fruit	9.1	12±1.2	3±0.2
Delonix regia	Gulmohar	Flower	12.3	11±0.2	5±0.7
Holarrhena antidysenterica	Kurchi	Bark	14.4	12±0.8	5±0.5
Lawsonia inermis	Mehndi	Leaves	8.7	09±0.2	3±0.8
Mangifera indica	Aam	Leaves	15.6	17±0.4	13±0.9
Psoralea corylifolia	Babchi	Seeds	12.2	15±0.5	14±0.6
Plumbago zeylanica		Root	7.1	17±0.6	5±0.3
Terminalia chebula	Harrad	Fruit	19.2	13±0.4	4±0.5
Trigonella foenum-graceum *	Methi	Leaves	10.5	14±0.8	14±0.3
Inactive extracts					
Acalypha indica	Kuppaimeni	Leaves	4.9		
Acorus calamus	Vacha	Rhizome	9.51		
Agave americana	Century plant	Leaves	11.3		
Aloe barbidens	Ghikuvar	Whole plant	22.0		
Allium sativum	Lesan	Leaves	13.3		
Areca catechu	Supari	Fruit	4.9		
Capsicum frutescens	Lal-mirch	Fruit	3.85		
Carum copticum	Ajwain	Seed	6.25		
Chicorium intybus	Chicory	Root	3.2		
Cassia fistula	Amaltas	Fruit	7.7		
Citrus sinensis	Musambi	Rind	5.9		
Curcuma longa	Haldi	Rhizome	15.2	No pigment inhibition activity	
Daucus carota	Carrot	Root	8.7		
Emblica officinalis	Amla	Fruit	7.2		
Foeniculum vulgare	Saunf	Seed	4.5		
Hedychium spicatum	Kapoor	Fruit	4.6		
Hemidesmus indicus	Ananthamool	Root	10.3		
Nigella sativa	Kalonji	Seed	7.6		
Nyctanthes arbor-tristis	Harshringar	Leaves	4.3		
Ocimum sanctum	Tulsi	Whole plant	7.2		
Piper cubeba	Kabab chini	Seeds	4.3		
Psidium guajava	Amrud	Leaves	9.4		
Punica granatum	Anar	Rind	15.3		
Sapindus trifoliatus	Ritha	Fruit	3.9		
Terminalia arjuna	Arjun	Bark	17.0		
Terminalia bellerica	Bahera	Fruit	14.3		
Zingiber officinale	Adrak	Rhizome	8.9		

* *T. foenum-graceum* enhanced the pigment production

Table 10: Pigment inhibitory activity of different fractions of *Mangifera indica* (leaves) extract

Name of the fraction	Concentration of extract (µg/ml)	Zone of inhibition against *C. violaceum* (CV12472) in mm		
		Total inhibition (r_1)	Growth inhibition (r_2)	Pigment inhibition (r_1-r_2)
Petroleum ether	200	-	-	-
	400	-	-	-
	800	-	-	-
	1600	-	-	-
Benzene	150	-	-	-
	300	-	-	-
	600	-	-	-
	1200	-	-	-
Ethyl acetate	225	-	-	-
	450	-	-	-
	900	-	-	-
	1800	5	-	5
Acetone	100	-	-	-
	200	-	-	-
	400	18	15	3
	800	21	16	5
Methanol	200	-	-	-
	400	15	-	15
	800	21	2	19
	1600	25	8	21

Data are the mean value of three experiments

- Shows no activity

Total inhibition= total zone of pigment inhibition including growth inhibition, if any

Table 11: Pigment inhibitory activity of different fractions of *Psoralea corylifolia* (seed) extract

Name of the fraction	Concentration of extract (µg/ml)	Zone of inhibition against *C. Violaceum* (CV12472) in mm		
		Total inhibition (r_1)	Growth inhibition (r_2)	Pigment inhibition (r_1-r_2)
Petroleum ether	200	-	-	-
	400	-	-	-
	800	-	-	-
	1600	-	-	-
Benzene	100	-	-	-
	200	-	-	-
	400	-	-	-
	800	-	-	-
Ethyl acetate	150	-	-	-
	300	-	-	-
	600	13	13	-
	1200	13	9	4
Acetone	100	-	-	-
	200	19	17	2
	400	25	21	4
	800	27	25	2
Methanol	200	-	-	-
	400	15	-	15
	800	16	-	16
	1600	18	3	15

Data are the mean value of three experiments

- Shows no activity

Total inhibition= total zone of pigment inhibition including growth inhibition, if any

Table 12: Interference in pigment production by different fractions of *Trigonella foenumgraecum* (seed) extract

Name of the fraction	Concentration of extract (µg/ml)	Zone of inhibition against *C. violaceum* (CV12472) in mm		
		Total interference (r_1)	Growth inhibition (r_2)	QS interference (r_1-r_2)
Petroleum ether	150	-	-	-
	300	-	-	-
	600	-	-	-
	1200	14	14	-
Benzene	100	-	-	-
	200	-	-	-
	400	-	-	-
	800	-	-	-
Ethyl acetate	300	-	-	-
	600	-	-	-
	900	11	11	-
	1800	17	17	-
Acetone	200	-	-	-
	400	-	-	-
	800	-	-	-
	1600	15	15	-
Methanol	150	-	-	-
	300	17	-	17
	600	20	-	20
	1200	24	9	15

Data are the mean value of three experiments

- Shows no activity

Total inhibition= total zone of pigment inhibition including growth inhibition, if any

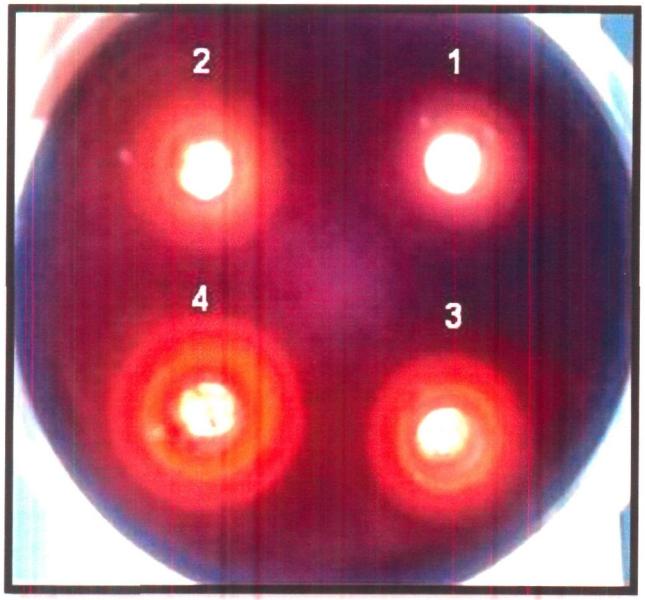


Plate 6: Inhibition of violacein by methanol extract of *M. indica* (leaf) in *C. violaceum* CV12472. 1). 200, 2). 400, 3). 800, 4). 1600 µg/ml.

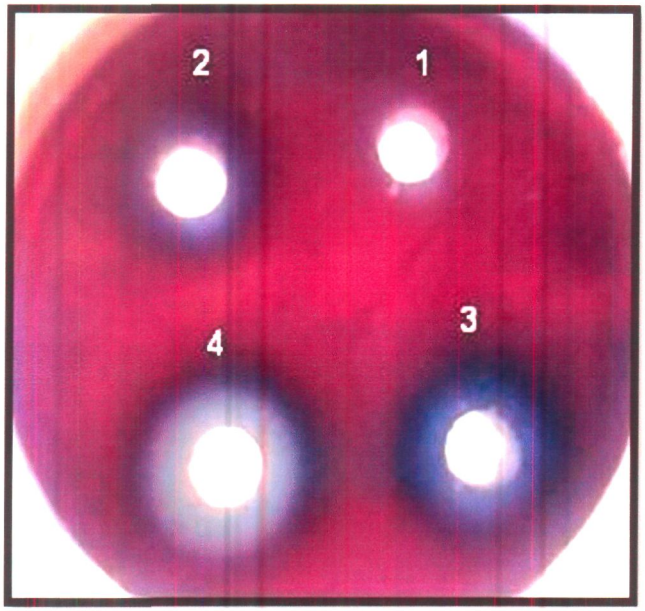


Plate 7: Increase of violacein production by methanol extract of *T. foenum-graceum* (seed) in *C. violaceum* CV12472. 1). 150, 2). 300, 3). 600, 4). 1200 µg/ml

Table 13: Screening of essential oils for their pigment inhibitory activity in *Chromobacterium violaceum* strains

Plant	Common name	Inhibition of violacein	
		CV12472	CV026
Active essential oils			
Cinnamomum verum	Cinnamon	12±0.1	5±0.4
Lavendula angustifolia	Lavender	10±0.3	4±0.2
Mentha piperita	Peppermint	13±0.2	11±0.2
Syzygium aromaticum	Clove	18±0.6	18±0.4
Inactive essential oils			
Apium graveolens	Celery	No pigment inhibition activity	
Citrus limon	Lemon		
Citrus paradisi	Grape		
Citrus sinensis	Orange		
Cymbopogon citratus	Lemongrass		
Cymbopogon martini	Palm rosa		
Eucalyptus sp.	Eucalyptus		
Foeniculum vulgare	Sweet fennel		
Myristica fragrans	Jaifal		
Olea europaea	Olive		
Petroselinum crispum	Parsley		
Rosmarinus officinalis	Rosemary		
Santalum album	sandalwood		
Thymus vulgaris	Thyme		
Trachyspermum ammi	Ajowan		
Zea mays	Corn		
Zingiber officinale	Ginger		

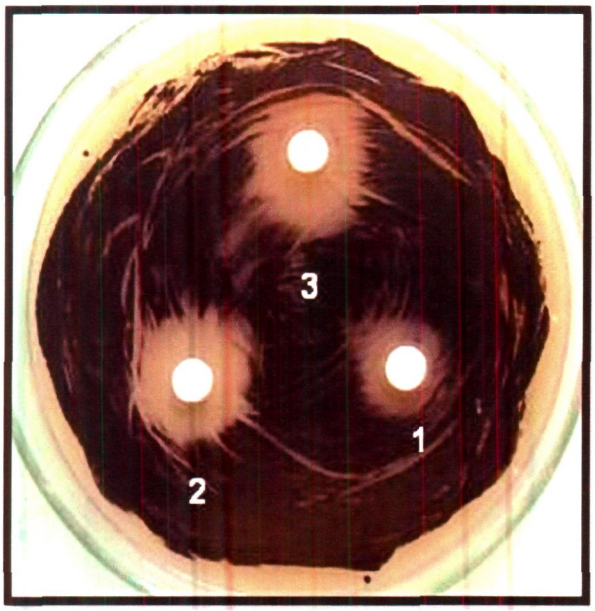


Plate 8: Inhibition of violacein by different concentrations of *Syzygium aromaticum* (clove) oil in *C. violaceum* CV12472. 1). 4 µl, 2). 8 µl, 3). 12 µl.

Table 14: Minimum inhibitory concentration of selected test agent against QS bacteria

Test agents	Minimum inhibitory concentration (µg/ml)			
	C. violaceum CVO26	*P. aeruginosa* PAO1	*P. aeruginosa* PAF-79	*A. hydrophila* WAF-38
Antibiotics				
Doxycycline	8	16	64	16
Ceftazidime	0.5	1	4	1
Medicinal plant extracts				
M. indica	1000	2000	2000	2000
T. foenum-graceum	1200	1200	2400	1200
P. corylifolia	750	1500	1250	1250
Essential oils				
Clove oil*	0.2	3.2	6.4	0.8
Peppermint oil*	1.6	6.4	3.2	1.6
Phytocompounds				
Eugenol*	1.5	1.5	6.25	3.12
Menthol	800	1600	800	400

*MIC values in % v/v

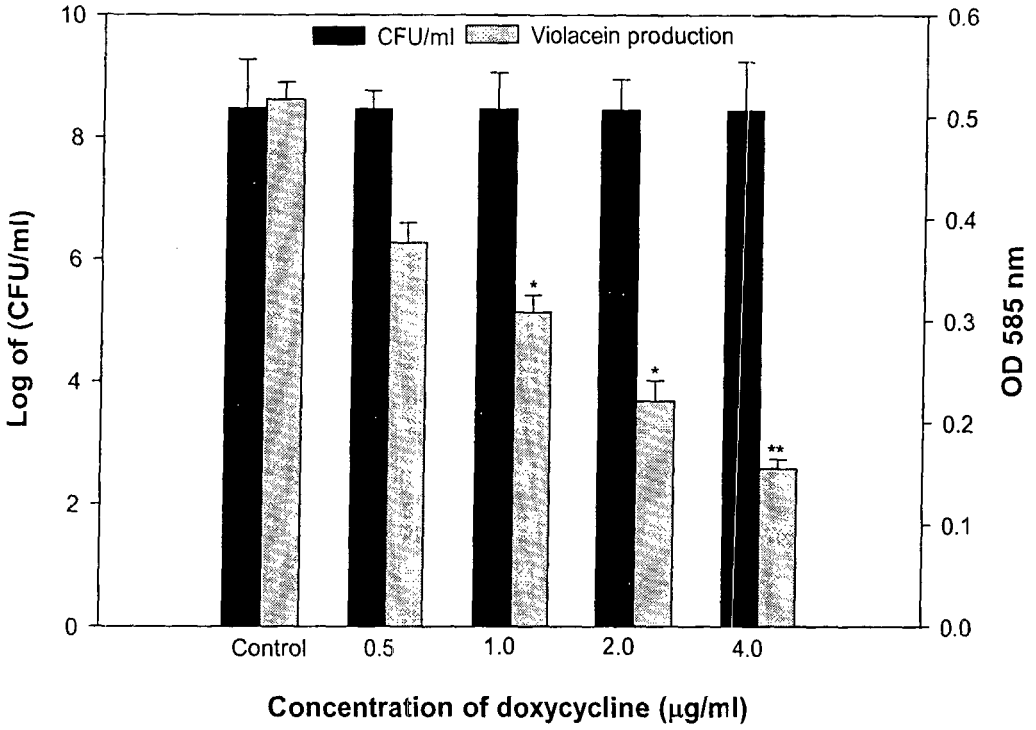


Figure 6: Quantitative assessment of violacein inhibition in CVO26 by sub-MICs of doxycycline.

Data are represented as percentage of violacein inhibition. All of the data are presented as mean ± SD. *, significance at $p \leq 0.05$, **, significance at $p \leq 0.005$

Table 15: Effect of sub-MICs of doxycyline on inhibition of quorum sensing regulated virulence factors in *P. aeruginosa* PAO1

Doxycycline concentration (ug/ml)	Elastase activity[a]	Total protease[b]	Pyocyanin production[c]	Chitinase activity[d]	EPS production[e]	Swarming motility[f]	Biofilm formation[g]
Control	0.167±0.029	1.253±0.029	5.15±0.38	0.106±0.018	1.25±0.055	82±4.5	0.317±0.020
0.5	0.143±0.011 (14.3)	0.799±0.018 (36.1)	2.70±0.34 (47.5)	0.090±0.008 (15)	0.87±0.011 (30.4)*	69±4.5 (15.8)	0.189±0.027 (40.3)*
1	0.107±0.011 (35.9)	0.628±0.025 (49.8)*	2.01±0.29 (60.9)*	0.077±0.009 (27.3)	0.740±0.049 (40.8)*	59±3.4 (28)*	0.151±0.023 (52.3)*
2	0.087±0.010 (47.9)*	0.585±0.021 (53.3)*	1.89±0.30 (63.3) *	0.058±0.011 (45.2)	0.540±0.020 (56.8)**	32±5.0 (60.9)*	0.133±0.022 (58.0)**
4	0.058±0.019 (65.2)*	0.501±0.010 (60.0)*	1.59±0.09 (69.1)**	0.032±0.012 (69.8)*	0.383±0.025 (69.6)**	22±2.8 (73.1)*	0.066±0.032 (79.1)**

[a]Elastase activity is expressed as the absorbance at OD_{495}.
[b]Total protease activity is expressed as the absorbance at OD_{600}.
[c]Pyocyanin concentrations were expressed as micrograms of pyocyanin produced per microgram of total protein.
[d]Chitinase activity is expressed as the absorbance at OD_{570}.
[e] EPS production is expressed as absorbance at OD_{480}
[f]Swarming motility is expressed as diameter of swarm in mm
[g]Biofilm formation is expressed as OD_{470} after incubation with crystal violet
The data represents mean values of three independent experiments. *, significance at $p \leq 0.05$, **, significance at $p \leq 0.005$, *** significance at $p \leq 0.001$
Values in the parentheses indicate percent reduction over control

Table 16: Effect of sub-MICs of doxycyline on inhibition of quorum sensing regulated virulence factors in *P. aeruginosa* PAF-79

Doxycycline concentration (ug/ml)	Elastase activity[a]	Total protease[b]	Pyocyanin production[c]	Chitinase activity[d]	EPS production[e]	Swarming motility[f]	Biofilm formation[g]
Control	0.114±0.012	0.930±0.034	3.73±0.27	0.091±0.019	1.55±0.014	55±2.0	0.444±0.036
4	0.098±0.006 (14)	0.747±0.041 (19.3)	2.31±0.23 (42.1)*	0.073±0.010 (19.7)	1.06±0.011 (31.6)	43±3.5 (21.8)*	0.223±0.027 (49.7)**
8	0.079±0.008 (30)	0.618±0.008 (33.5)	2.03±0.29 (45.5)*	0.059±0.021 (35.1)	0.673±0.025 (56.5)*	35±3.0 (36.3)*	0.167±0.012 (62.3)**
16	0.060±0.006 (47.3)*	0.441±0.026 (52.5)*	1.80±0.30 (51.7)*	0.038±0.010 (58.2)*	0.593±0.025 (61.7)**	29±3.5 (47.2)**	0.137±0.013 (69.1)**
32	0.046±0.006 (59.6)*	0.352±0.014 (62.1)*	1.47±0.09 (60.5)*	0.023±0.006 (74.7)*	0.473±0.038 (69.4)**	20±1.0 (63.6)**	0.119±0.017 (73.1)**

[a]Elastase activity is expressed as the absorbance at OD_{495}.
[b]Total protease activity is expressed as the absorbance at OD_{600}.
[c]Pyocyanin concentrations were expressed as micrograms of pyocyanin produced per microgram of total protein.
[d]Chitinase activity is expressed as the absorbance at OD_{570}.
[e] EPS production is expressed as absorbance at OD_{480}
[f]Swarming motility is expressed as diameter of swarm in mm
[g]Biofilm formation is expressed as OD_{470} after incubation with crystal violet
The data represents mean values of three independent experiments. *, significance at $p \leq 0.05$, **, significance at $p \leq 0.005$, *** significance at $p \leq 0.001$
Values in the parentheses indicate percent reduction over control

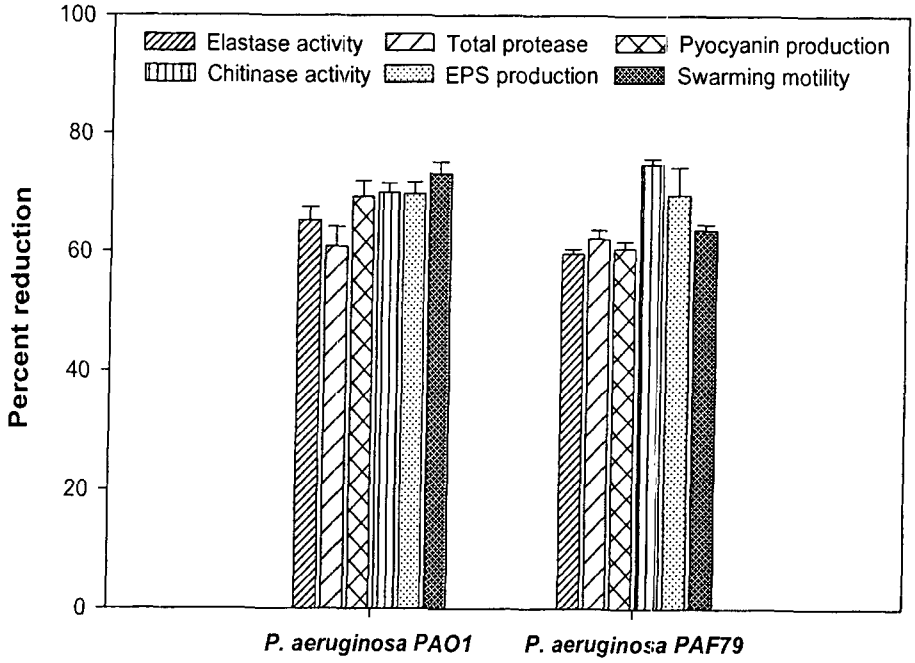


Figure 7: Effect of doxycycline on quorum sensing regulated factors in *P. aeruginosa* PAO1 and *P. aeruginosa* PAF79 at 4 and 32 µg/ml respectively

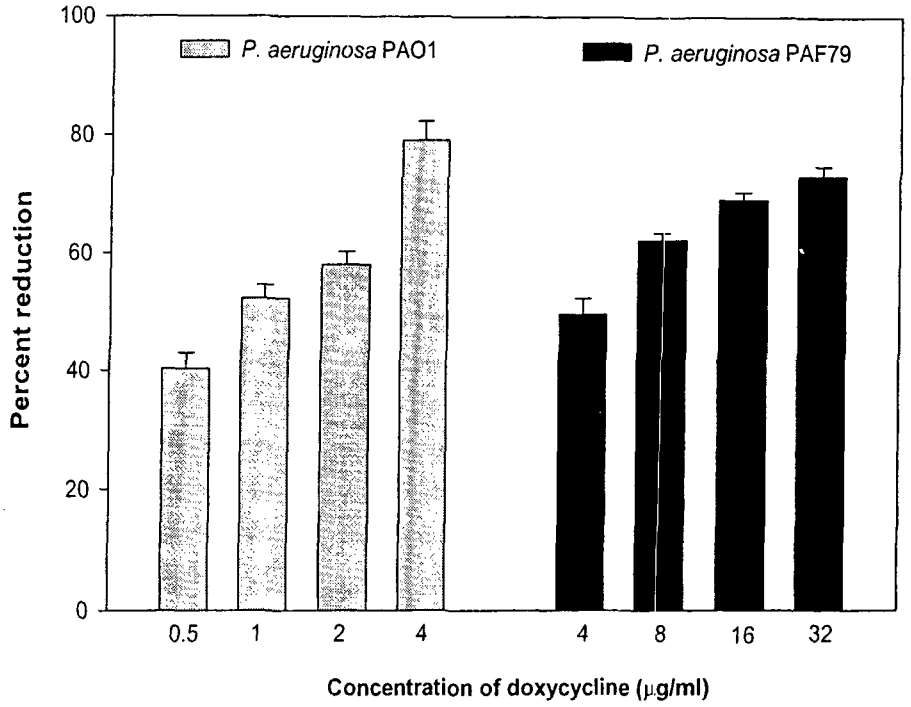


Figure 8: Effect of doxycycline on biofilm formation in *P. aeruginosa* PAO1 and *P. aeruginosa* PAF79 at respective sub-MICs

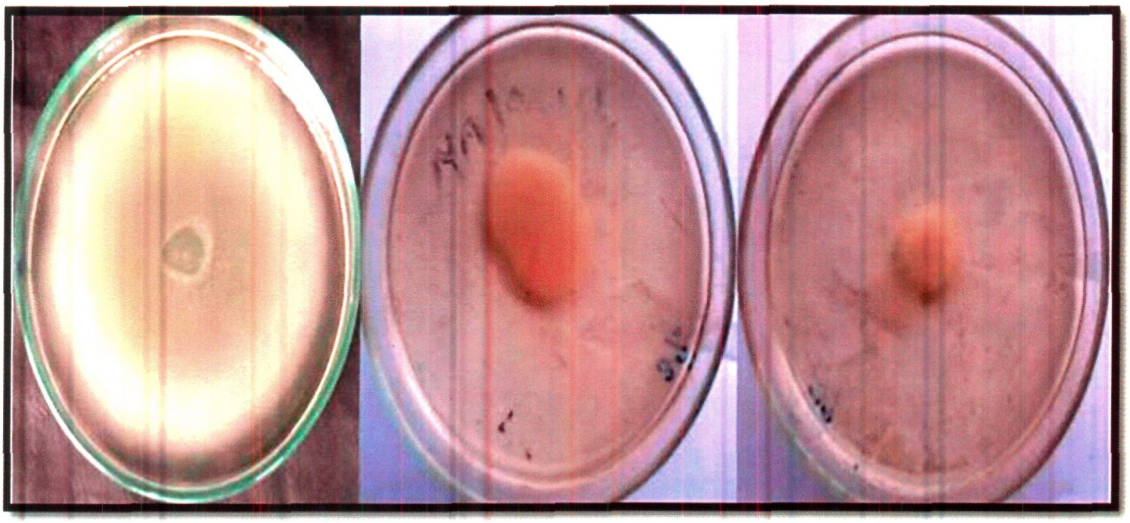


Plate 9: Inhibition of swarming motility in *P. aeruginosa* PAO1 by sub-MICs of doxycycline, A). Untreated control; B). 2 µg/ml; C). 4µg/ml

Table 17: Effect of sub-MICs of doxycycline on inhibition of quorum sensing regulated virulence factors in *Aeromonas hydrophila* WAF-38

Concentration (μg/ml)	Total protease[a]	EPS production[b]	Biofilm formation[c]
Control	0.618±0.045	0.594±0.015	0.239±0.016
1	0.564±0.032 (08.7)	0.525±0.038 (11.6)	0.188±0.015 (21.3)
2	0.405±0.028 (34.4)	0.449±0.022 (24.4)	0.172±0.002 (28.0)
4	0.271±0.041 (56.1)*	0.399±0.008 (32.8)	0.091±0.018 (61.9)**
8	0.204±0.024 (66.9)*	0.291±0.017 (51.0)*	0.068±0.001 (71.5)**

[a] Total protease activity is expressed as the absorbance at OD_{600}.
[b] EPS production is expressed as absorbance at OD_{480}
[c] Biofilm formation is expressed as OD_{470} after incubation with crystal violet.
The data represents mean values of three independent experiments. *, significance at $p \leq 0.05$, **, significance at $p \leq 0.005$, *** significance at $p \leq 0.001$
Values in the parentheses indicate percent reduction over control

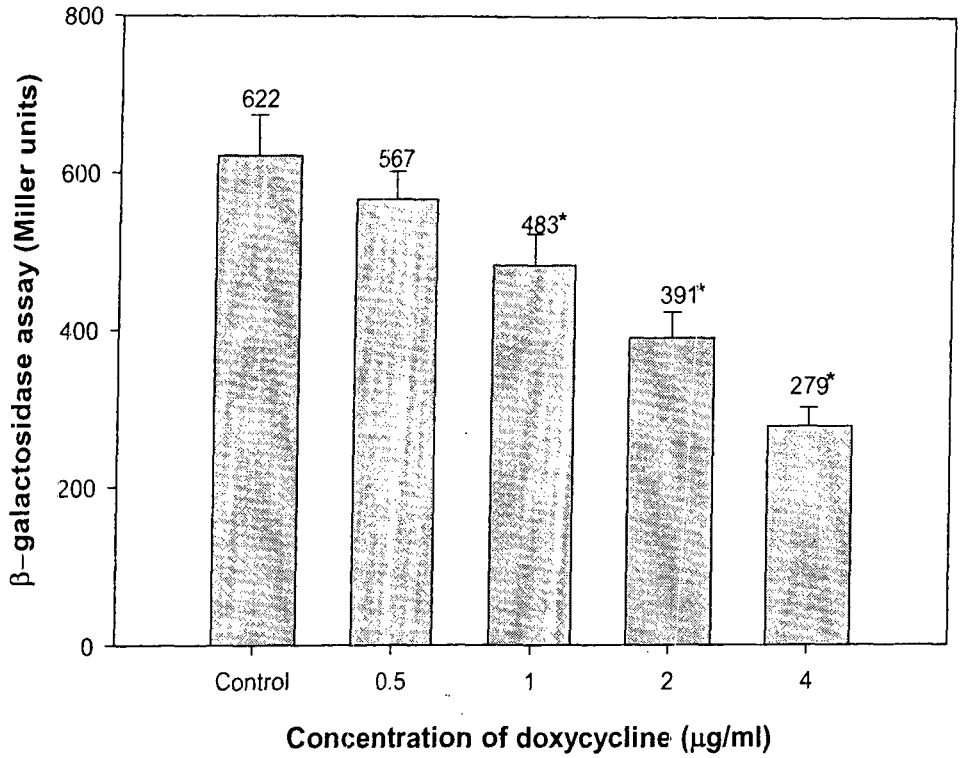


Figure 9: Effect of sub-MICs of doxycycline on β-galactosidase activity in *E. coli* MG4/pKDT17
All of the data are presented as mean ± SD. *, significance at $p \leq 0.05$

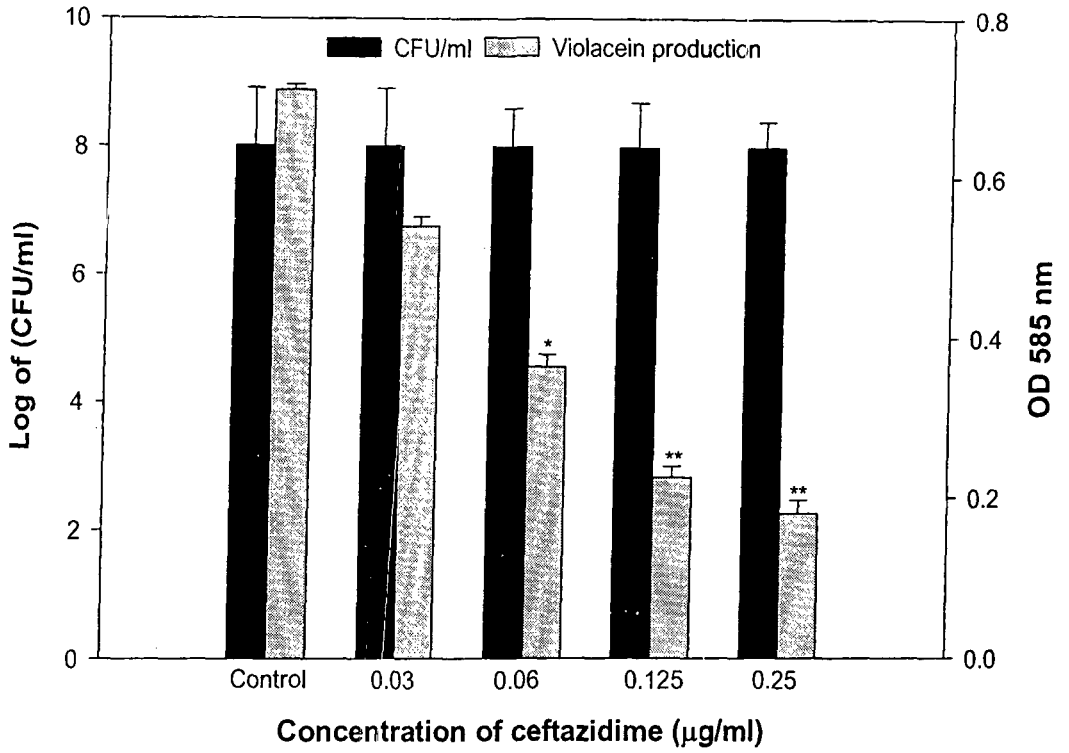


Figure 10: Quantitative assessment of violacein inhibition in CVO26 by sub-MICs of ceftazidime.

Data are represented as percentage of violacein inhibition. All of the data are presented as mean ± SD. *, significance at $p \leq 0.05$, **, significance at $p \leq 0.005$

Table 18: Effect of sub-MICs of ceftazidime on inhibition of quorum sensing regulated virulence factors in *P. aeruginosa* PAO1

Ceftazidime concentration (ug/ml)	Elastase activity[a]	Total protease[b]	Pyocyanin production[c]	Chitinase activity[d]	EPS production[e]	Swarming motility[f]	Biofilm formation[g]
Control	0.167±0.029	1.253±0.029	5.15±0.38	0.105±0.020	1.25±0.055	82.00 ± 2.0	0.317±0.020
0.06	0.149±0.033 (10.7)	0.997±0.032 (20.4)	4.4±0.12 (14.5)	0.089±0.004 (15.2)	1.03±0.043 (17.6)	31.00 ± 2.5(62.1)**	0.287±0.019 (9.4)
0.125	0.097±0.018 (41.9)	0.800±0.015 (36.1)	4.2±0.36 (18.4)	0.069±0.016 (34.2)	0.743±0.024 (40.5)	25.00 ± 2.5(69.5)**	0.205±0.031 (35.3)
0.25	0.075±0.008 (55.0)*	0.625±0.009 (50.1)*	3.17±0.19 (38.4)*	0.056±0.021 (46.6)*	0.596±0.031 (52.3)*	18.00 ± 1.0(78.0)***	0.176±0.026 (44.4)*
0.5	0.062±0.022 (62.8)*	0.554±0.013 (55.7)*	2.0±0.21 (61.1)*	0.038±0.028 (63.8)*	0.513±0.027 (58.9)*	15.00 ± 1.5(81.7)**	0.095±0.021 (70.0)**

[a]Elastase activity is expressed as the absorbance at OD_{495}.
[b]Total protease activity is expressed as the absorbance at OD_{600}.
[c]Pyocyanin concentrations were expressed as micrograms of pyocyanin produced per microgram of total protein.
[d]Chitinase activity is expressed as the absorbance at OD_{570}.
[e] EPS production is expressed as absorbance at OD_{480}
[f]Swarming motility is expressed as diameter of swarm in mm
[g]Biofilm formation is expressed as OD_{470} after incubation with crystal violet
The data represents mean values of three independent experiments. *, significance at $p \leq 0.05$, **, significance at $p \leq 0.005$, *** significance at $p \leq 0.001$
Values in the parentheses indicate percent reduction over control

Table 19: Effect of sub-MICs of ceftazidime on inhibition of quorum sensing regulated virulence factors in *P. aeruginosa* PAF-79

Ceftazidime concentration (ug/ml)	Elastase activity[a]	Total protease[b]	Pyocyanin production[c]	Chitinase activity[d]	EPS production[e]	Swarming motility[f]	Biofilm formation[g]
Control	0.114±0.012	0.930±0.034	3.73±0.27	0.091±0.019	1.55±0.014	55±2.0	0.444±0.036
0.25	0.072±0.006 (36.8)*	0.843±0.024 (9.3)	3.5±0.3 (6.1)	0.083±0.010 (8.7)	1.23±0.067 (20.6)	38.00 ± 1.2 (30.9)	0.303±0.021 (31.7)
0.5	0.059±0.012 (48.2)*	0.699±0.039 (24.8)	3.14±0.18 (15.8)	0.071±0.006 (21.9)	0.888±0.037 (42.7)	29.00 ± 1.0 (47.2)*	0.273±0.039 (38.5)
1.0	0.052±0.010 (54.3)*	0.571±0.018 (38.6)	2.65±0.11 (28.9)	0.058±0.009 (36.2)	0.732±0.028 (52.7)*	20.00 ± 0.5 (63.6)**	0.210±0.014 (52.7)*
2.0	0.039±0.004 (65.7)**	0.491±0.026 (47.2)*	1.84±0.21 (50.6)*	0.041±0.003 (54.9)*	0.616±0.042 (60.2)*	13.00 ± 2.0 (76.6)**	0.154±0.021 (65.3)**

[a]Elastase activity is expressed as the absorbance at OD_{495}.
[b]Total protease activity is expressed as the absorbance at OD_{600}.
[c]Pyocyanin concentrations were expressed as micrograms of pyocyanin produced per microgram of total protein.
[d]Chitinase activity is expressed as the absorbance at OD_{570}.
[e] EPS production is expressed as absorbance at OD_{480}
[f]Swarming motility is expressed as diameter of swarm in mm
[g]Biofilm formation is expressed as OD_{470} after incubation with crystal violet
The data represents mean values of three independent experiments. *, significance at $p \leq 0.05$, **, significance at $p \leq 0.005$, *** significance at $p \leq 0.001$
Values in the parentheses indicate percent reduction over control

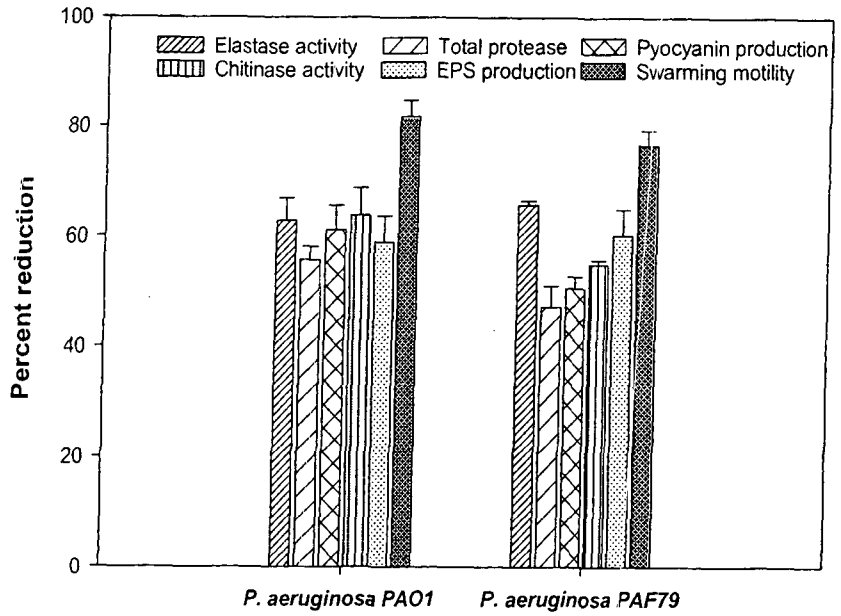


Figure 11: Effect of ceftazidime on quorum sensing regulated factors in *P. aeruginosa* PAO1 and *P. aeruginosa* PAF79 at 0.5 and 2 μg/ml respectively

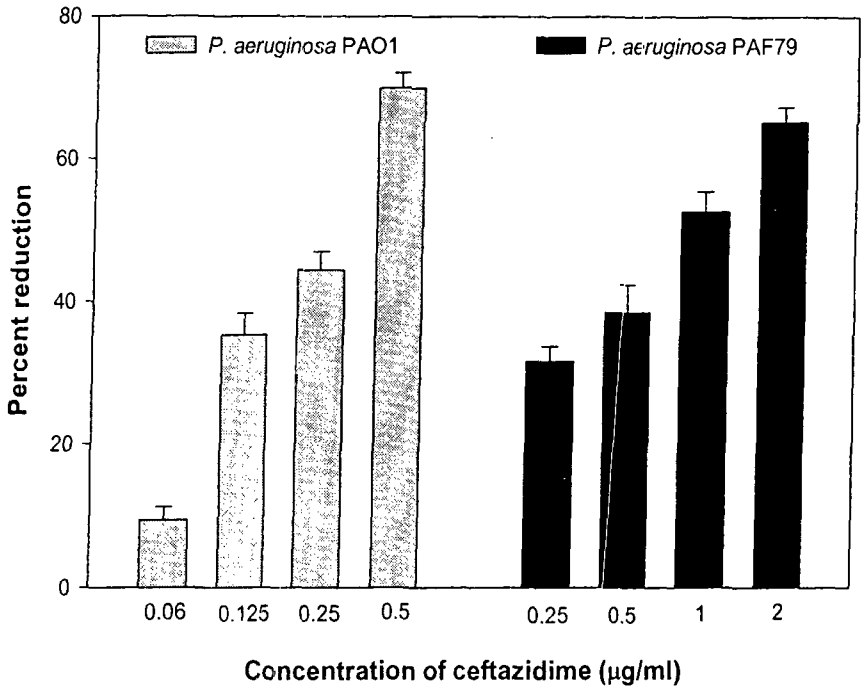


Figure 12: Effect of ceftazidime on biofilm formation in *P. aeruginosa* PAO1 and *P. aeruginosa* PAF79 at respective sub-MICs

Table 20: Effect of sub-MICs of ceftazidime on inhibition of quorum sensing regulated virulence factors in *Aeromonas hydrophila* WAF-38

Ceftazidime concentration (µg/ml)	Total protease[a]	EPS production[b]	Biofilm formation[c]
Control	0.618±0.045	0.594±0.015	0.239±0.016
0.062	0.528±0.036 (14.5)	0.550±0.033 (07.4)	0.165±0.008 (30.9)
0.125	0.452±0.031 (26.8)	0.469±0.016 (20.3)	0.150±0.010 (37.2)
0.25	0.309±0.040 (50.0)*	0.369±0.025 (37.8)	0.117±0.020 (51.0)*
0.5	0.266±0.019 (56.9)*	0.236±0.011 (60.2)**	0.083±0.010 (65.2)**

[a] Total protease activity is expressed as the absorbance at OD_{600}.
[b] EPS production is expressed as absorbance at OD_{480}
[c] Biofilm formation is expressed as OD_{470} after incubation with crystal violet.
The data represents mean values of three independent experiments. *, significance at $p \leq 0.05$, **, significance at $p \leq 0.005$, *** significance at $p \leq 0.001$
Values in the parentheses indicate percent reduction over control

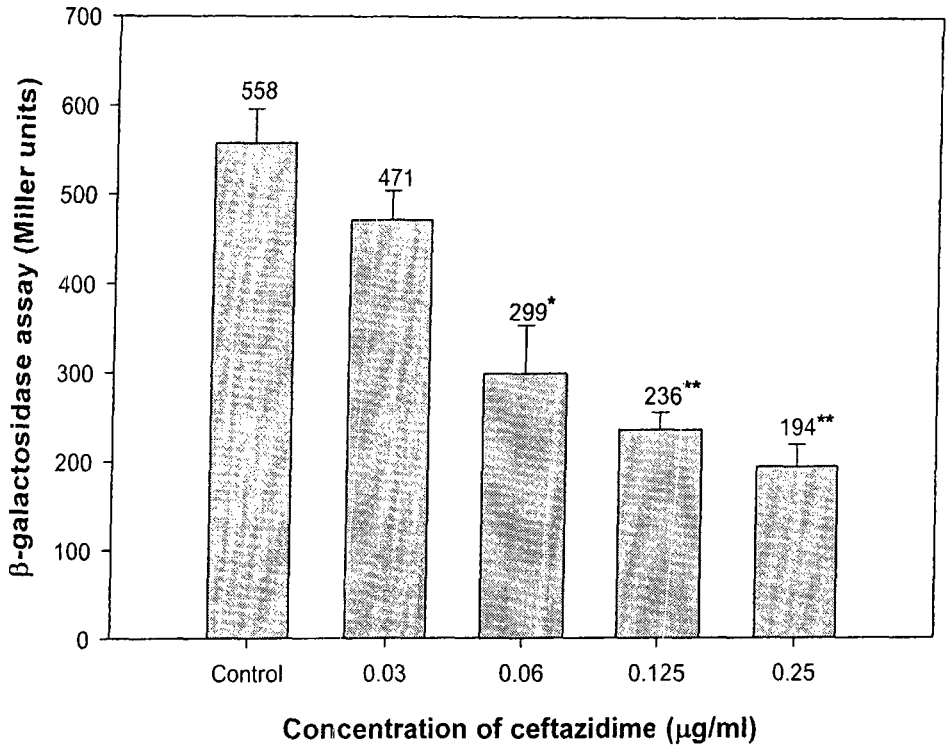


Figure 13: Effect of sub-MICs of ceftazidime on β-galactosidase activity in *E. coli* MG4/pKDT17.
All of the data are presented as mean ± SD. *, significance at $p \leq 0.05$, **, significance at $p \leq 0.005$

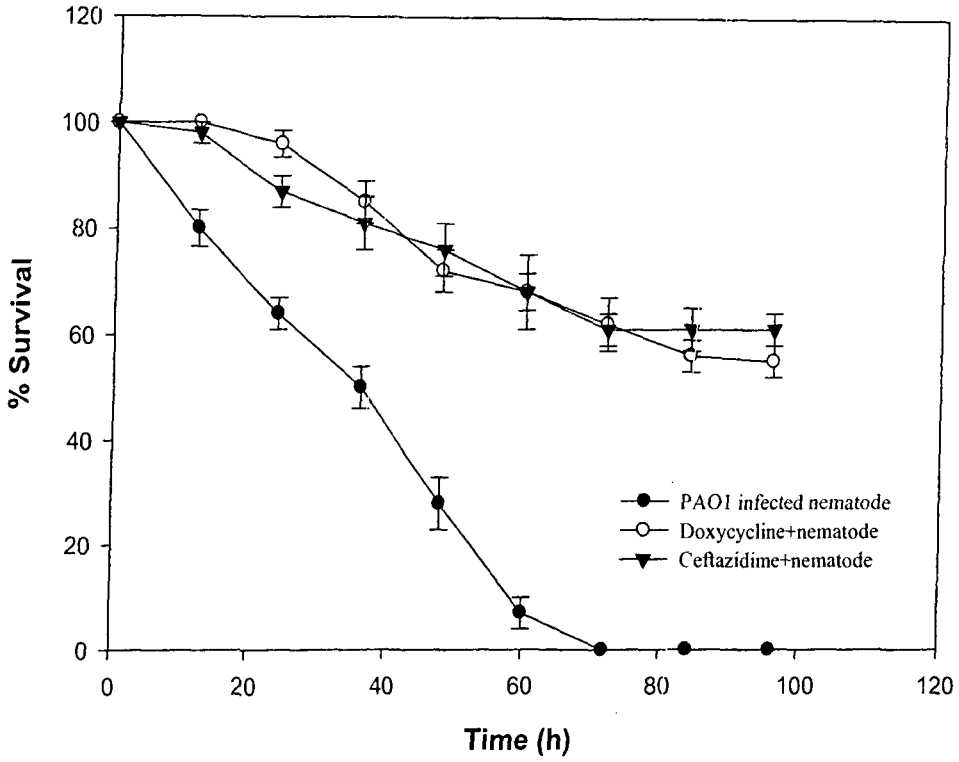


Figure 14: Anti-infection potential of doxycycline and ceftazidime at respective sub-MICs (4 and 0.25 µg/ml) in preinfected *C. elegans* nematode model.
Means values of triplicate independent experiments and SDs are shown.

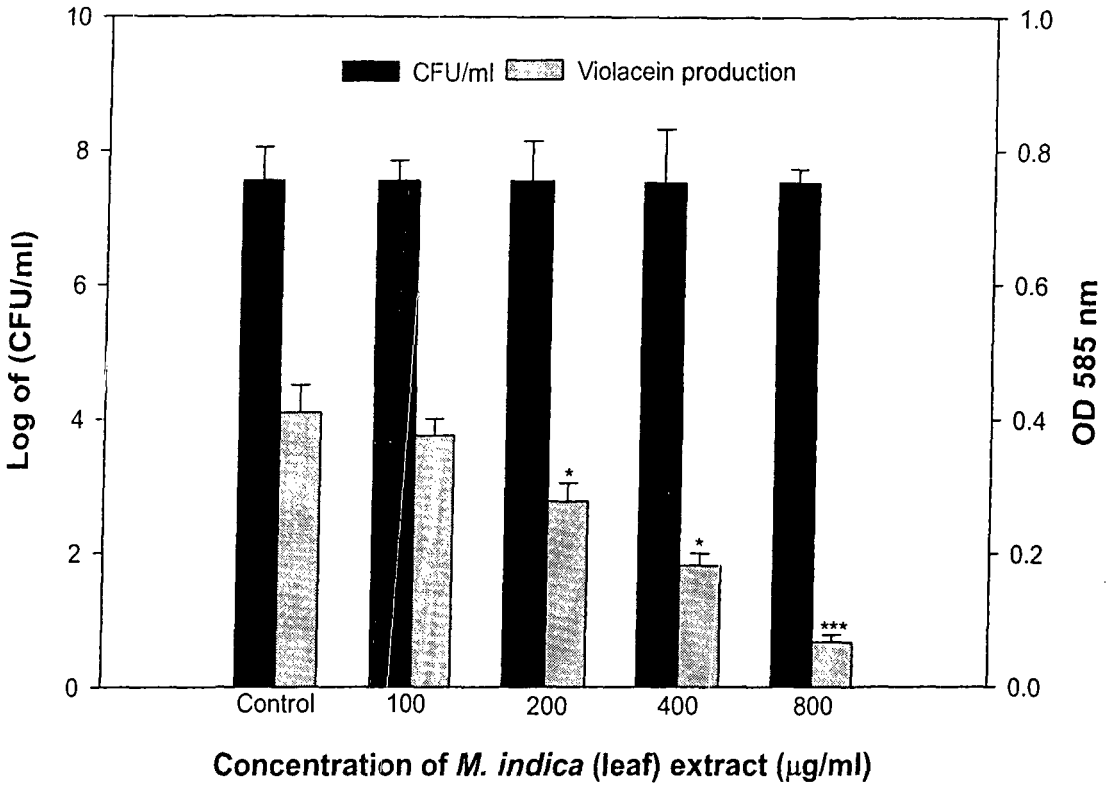


Figure 15: Quantitative assessment of violacein inhibition in CVO26 by sub-MICs of *M. indica* (leaf) extract.
All of the data are presented as mean ± SD. *, significance at $p \leq 0.05$, **, significance at $p \leq 0.005$, ***, significance at $p \leq 0.001$

Table 21: Effect of sub-MICs of methanolic extract of *Mangifera indica* (leaf) on inhibition of quorum sensing regulated virulence factors in *P. aeruginosa* PAO1

Extract concentration (µg/ml)	Elastase activity[a]	Total protease[b]	Pyocyanin production[c]	Chitinase activity[d]	EPS production[e]	Swarming Motility[f]	Biofilm formation[g]
Control	0.181±0.044	1.091±0.035	4.4±0.34	0.141±0.019	1.110±0.024	72±1.5	0.406±0.039
100	0.149±0.032 (17.6)	0.857±0.028 (21.4)	1.33±0.33 (69.7)**	0.110±0.020 (21.9)	0.678±0.018 (38.9)	53±2.0(26.3)	0.256±0.027 (36.9)
200	0.110±0.019 (39.2)*	0.613±0.025 (43.8)*	0.87±0.20 (80.2)**	0.09±0.005 (36.1)	0.601±0.019 (45.8)	39±2.0 (45.8)*	0.210±0.023 (48.2)*
400	0.090±0.021 (50.2)*	0.547±0.016 (49.8)*	0.63±0.033 (85.6)***	0.081±0.009 (42.5)	0.552±0.010 (50.2)*	31±1.1 (56.9)*	0.179±0.031 (55.9)*
800	0.043±0.017 (76.2)**	0.479±0.017 (56.0)*	0.49±0.015 (88.8)***	0.063±0.012 (55.3)*	0.462±0.015 (58.3)*	20±1.7 (72.2)**	0.112±0.019 (72.4)**

[a]Elastase activity is expressed as the absorbance at OD_{495}.
[b]Total protease activity is expressed as the absorbance at OD_{600}.
[c]Pyocyanin concentrations were expressed as micrograms of pyocyanin produced per microgram of total protein.
[d]Chitinase activity is expressed as the absorbance at OD_{570}.
[e] EPS production is expressed as absorbance at OD_{480}
[f]Swarming motility is expressed as diameter of swarm in mm
[g]Biofilm formation is expressed as OD_{470} after incubation with crystal violet
The data represents mean values of three independent experiments. *, significance at $p \leq 0.05$, **, significance at $p \leq 0.005$, *** significance at $p \leq 0.001$
Values in the parentheses indicate percent reduction over control

Table 22: Effect of sub-MICs of methanolic extract of *Mangifera indica* (leaf) on inhibition of quorum sensing regulated virulence factors in *P. aeruginosa* PAF-79

Extract concentration (µg/ml)	Elastase activity[a]	Total protease[b]	Pyocyanin production[c]	Chitinase activity[d]	EPS production[e]	Swarming Motility[f]	Biofilm formation[g]
Control	0.167±0.025	1.039±0.041	3.8±0.25	0.139±0.005	0.886±0.036	48±1.5	0.331±0.027
125	0.082±0.022 (50.8)*	0.441±0.033 (57.5)*	3.1±0.16 (18.4)	0.054±0.026 (61.1)**	0.754±0.011 (14.8)	26±2 (45.8)*	0.226±0.018 (31.7)
250	0.070±0.017 (58)**	0.281±0.015 (72.9)**	2.2±0.10 (42.1)	0.041±0.010 (70.5)**	0.748±0.023 (15.5)	20±1.5 (58.3)**	0.173±0.015 (47.7)*
500	0.065±0.017 (61)*	0.256±0.027 (75.3)**	2.1±0.23 (44.7)*	0.038±0.011 (72.6)**	0.420±0.044 (52.5)*	18±1.2 (62.5)**	0.124±0.024 (61.9)*
1000	0.046±0.017 (73.6)**	0.177±0.010 (82.9)***	1.3±0.041 (65.7)*	0.032±0.006 (76.9)**	0.379±0.029 (57.2)*	14±1 (70.3)**	0.086±0.005 (74)**

[a]Elastase activity is expressed as the absorbance at OD_{495}.
[b]Total protease activity is expressed as the absorbance at OD_{600}.
[c]Pyocyanin concentrations were expressed as micrograms of pyocyanin produced per microgram of total protein.
[d]Chitinase activity is expressed as the absorbance at OD_{570}.
[e] EPS production is expressed as absorbance at OD_{480}
[f]Swarming motility is expressed as diameter of swarm in mm
[g]Biofilm formation is expressed as OD_{470} after incubation with crystal violet
The data represents mean values of three independent experiments. *, significance at $p \leq 0.05$, **, significance at $p \leq 0.005$, *** significance at $p \leq 0.001$
Values in the parentheses indicate percent reduction over control

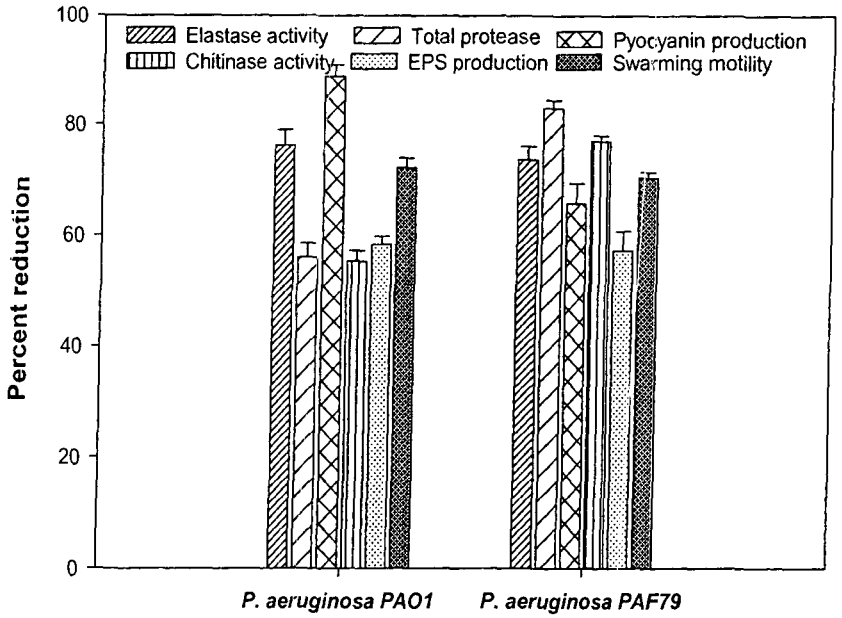


Figure 16: Effect of *M. indica* (leaf) extract on quorum sensing regulated factors in *P. aeruginosa* PAO1 and *P. aeruginosa* PAF79 at 800 and 1000 µg/ml respectively

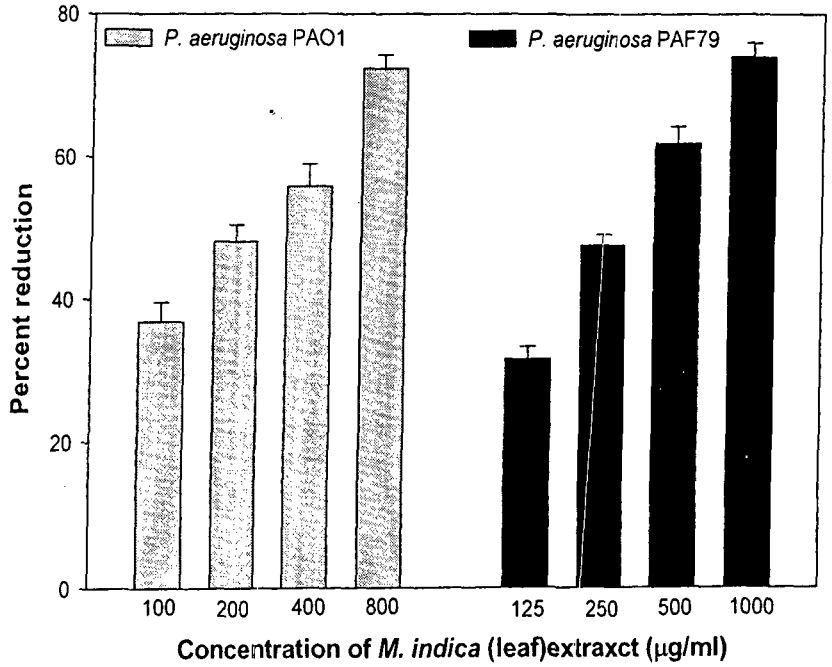


Figure 17: Effect of *M. indica* (leaf) extract on biofilm formation in *P. aeruginosa* PAO1 and *P. aeruginosa* PAF79 at respective sub-MICs

Table 23: Effect of sub-MICs of methanolic extract of *Mangifera indica* (leaf) on inhibition of quorum sensing regulated virulence factors in *Aeromonas hydrophila* WAF-38

Concentration (µg/ml)	Total protease[a]	EPS production[b]	Biofilm formation[c]
Control	0.589±0.051	0.748±0.021	0.226±0.006
125	0.271±0.015 (53.9)*	0.661±0.027 (11.6)	0.099±0.004 (56.1)*
250	0.223±0.006 (62.1)*	0.434±0.009 (41.9)*	0.087±0.009 (61.5)*
500	0.2±0.010 (66.0)**	0.357±0.005 (52.2)*	0.054±0.006 (76.1)**
1000	0.182±0.004 (69.1)**	0.306±0.018 (59.0)*	0.04±0.003(82.3)***

[a] Total protease activity is expressed as the absorbance at OD_{600}.
[b]EPS production is expressed as absorbance at OD_{480}
[c] *Biofilm formation is expressed as OD_{470} after incubation with crystal violet.*
The data represents mean values of three independent experiments. *, significance at $p \leq 0.05$, **, significance at $p \leq 0.005$, *** significance at $p \leq 0.001$
Values in the parentheses indicate percent reduction over control

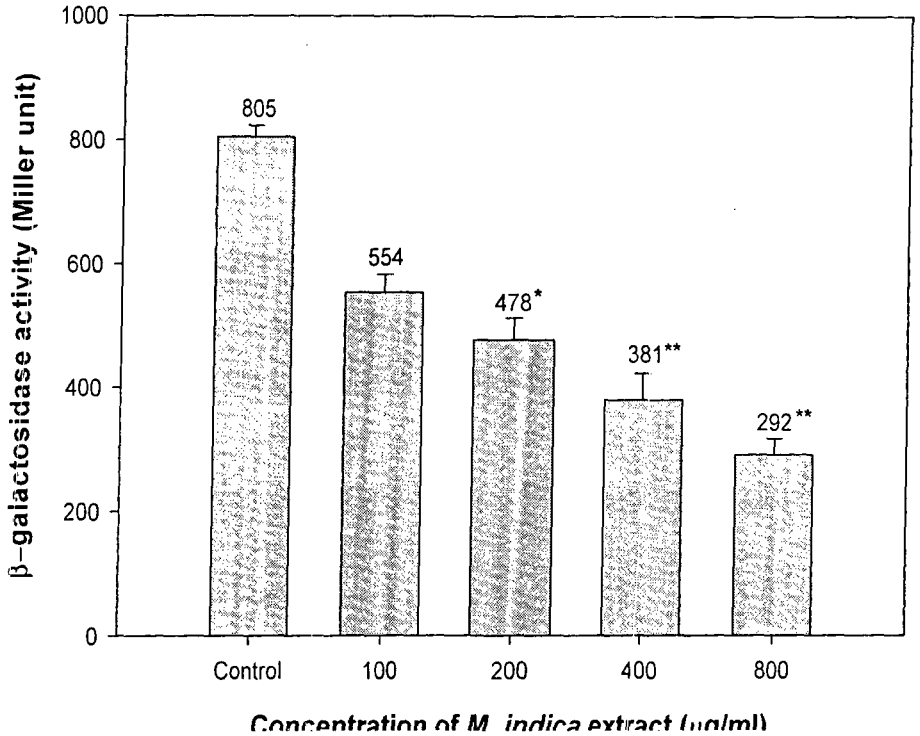


Figure 18: Effect of *M. indica* (leaf) extract on β-galactosidase activity in *E. coli* MG4/pKDT17.
All of the data are presented as mean ± SD. *, significance at $p \leq 0.05$, **, significance at $p \leq 0.005$

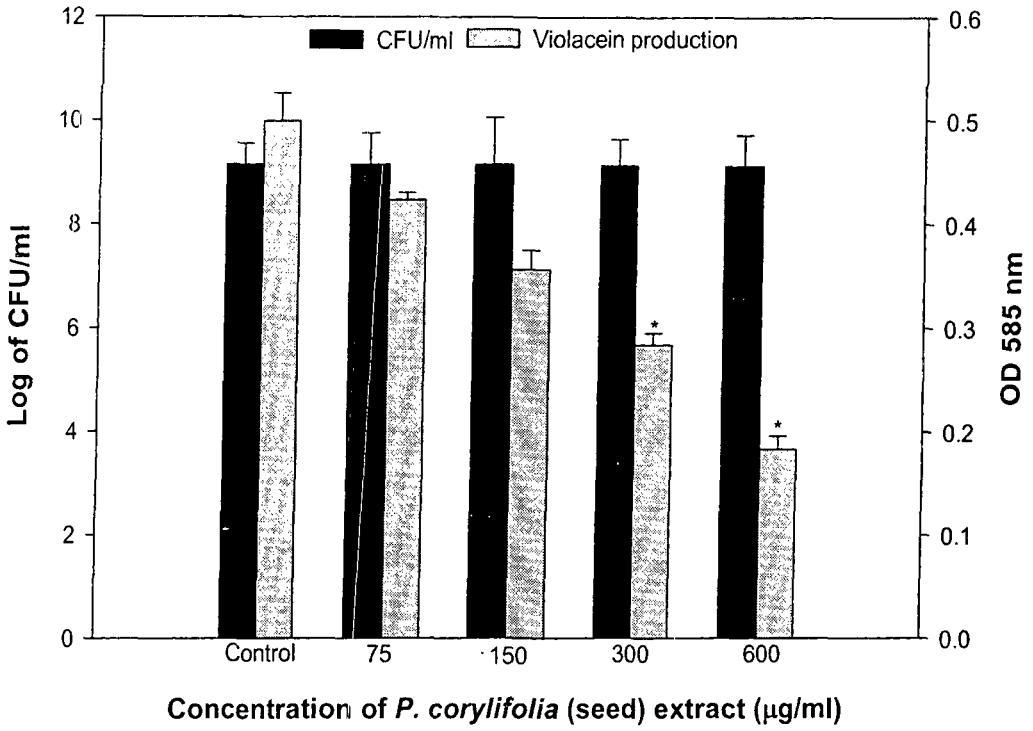


Figure 19: Quantitative assessment of violacein inhibition in CVO26 by sub-MICs of *P. corylifolia* (seed) extract.
All of the data are presented as mean ± SD. *, significance at $p \leq 0.05$

Table 24: Effect of sub-MICs of methanolic extract of *Psoralea corylifolia* (seed) on inhibition of quorum sensing regulated virulence factors in *P. aeruginosa* PAO1

Extract concentration (µg/ml)	Elastase activity[a]	Total protease[b]	Pyocyanin production[c]	Chitinase activity[d]	EPS production[e]	Swarming motility[f]	Biofilm formation[g]
Control	0.181±0.044	1.420±0.038	5.2±0.6	0.120±0.009	0.991±0.045	72±1.5	0.406±0.039
125	0.156±o.021 (13.8)	1.075±0.036 (24.2)	2.45±0.19 (52.8)*	0.082±0.011 (31.6)	0.754±0.049 (23.9)	46±1.45 (36.1)	0.312±0.028 (23.1)
250	0.115±0.013 (36.4)	1.01±0.025 (28.8)	1.72±0.33 (66.9)**	0.048±0.017 (60)**	0.700±0.018 (29.3)	37±2.0 (48.6)*	0.186±0.026 (54.1)**
500	0.101±0.006 (44.1)	0.938±0.019 (33.9)	1.5±0.22 (71.1)**	0.040±0.005 (66.6)**	0.515±0.027 (48.0)*	29±0.80 (59.7)*	0.119±0.031 (70.6)***
1000	0.091±0.009 (49.7)*	0.801±0.007 (43.5)*	0.69±0.10 (86.7)***	0.029±0.005 (75.8)***	0.429±0.025 (56.7)*	26±1.2 (63.8)*	0.085±0.026 (79.0)**

[a]Elastase activity is expressed as the absorbance at OD_{495}.
[b]Total protease activity is expressed as the absorbance at OD_{600}.
[c]Pyocyanin concentrations were expressed as micrograms of pyocyanin produced per microgram of total protein.
[d]Chitinase activity is expressed as the absorbance at OD_{570}.
[e] EPS production is expressed as absorbance at OD_{480}
[f]Swarming motility is expressed as diameter of swarm in mm
[g]Biofilm formation is expressed as OD_{470} after incubation with crystal violet
The data represents mean values of three independent experiments. *, significance at $p \leq 0.05$, **, significance at $p \leq 0.005$, *** significance at $p \leq 0.001$
Values in the parentheses indicate percent reduction over control

Table 25: Effect of sub-MICs of methanolic extract of *Psoralea corylifolia* (seed) on inhibition of quorum sensing regulated virulence factors in *P. aeruginosa* PAF-79

Extract concentration (µg/ml)	Elastase activity[a]	Total protease[b]	Pyocyanin production[c]	Chitinase activity[d]	EPS production[e]	Swarming motility[f]	Biofilm formation[g]
Control	0.167±0.025	1.039±0.041	3.8±0.25	0.139±0.005	0.886±0.036	48±1.5	0.331±0.027
100	0.148±0.004 (11.3)	0.938±0.021 (9.7)	3±0.2 (21)	0.114±0.008 (17.9)	0.661±0.015 (25.3)	40±0.5 (16.6)	0.292±0.014 (11.7)
200	0.140±0.015 (16.1)	0.891±0.030 (14.2)	2.4±0.13 (36.8)	0.07±0.005 (49.6)*	0.525±0.018 (40.6)	33±2 (31.2)*	0.217±0.003 (34.4)
400	0.115±0.007 (31.1)	0.748±0.014 (28)	2.1±0.082 (44.7)*	0.063±0.007 (54.6)*	0.373±0.012 (57.9)*	24±1.5 (50)*	0.177±0.017 (46.5)
800	0.090±0.003 (46.1)*	0.515±0.012 (50.5)*	1.6±0.054 (57.8)*	0.051±0.008 (63.3)*	0.292±0.014 (67)*	22±2.5 (54.1)*	0.094±0.008 (71.6)**

[a]Elastase activity is expressed as the absorbance at OD_{495}.
[b]Total protease activity is expressed as the absorbance at OD_{600}.
[c]Pyocyanin concentrations were expressed as micrograms of pyocyanin produced per microgram of total protein.
[d]Chitinase activity is expressed as the absorbance at OD_{570}.
[e] EPS production is expressed as absorbance at OD_{480}
[f]Swarming motility is expressed as diameter of swarm in mm
[g]Biofilm formation is expressed as OD_{470} after incubation with crystal violet
The data represents mean values of three independent experiments. *, significance at $p \leq 0.05$, **, significance at $p \leq 0.005$, *** significance at $p \leq 0.001$
Values in the parentheses indicate percent reduction over control

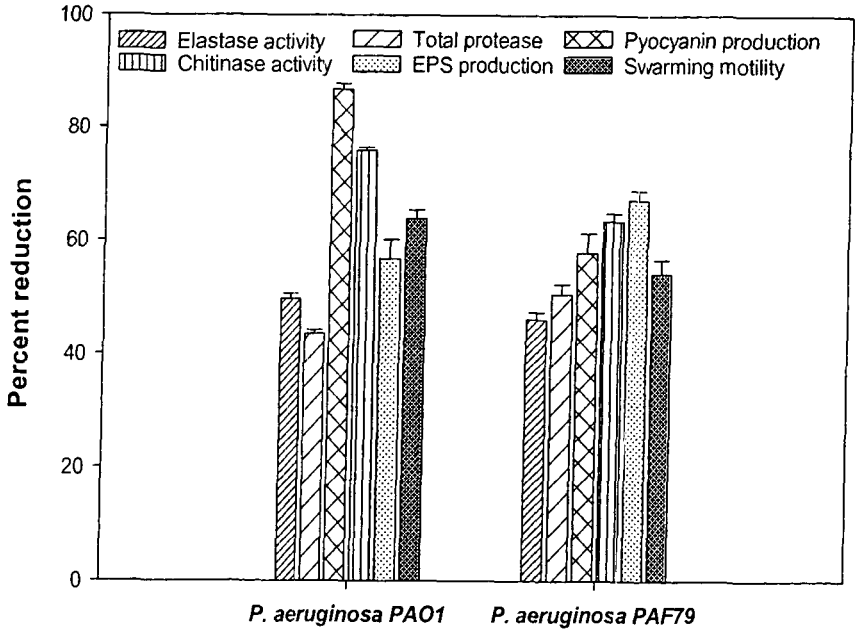


Figure 20: Effect of *P. corylifolia* (seed) extract on quorum sensing regulated factors in *P. aeruginosa* PAO1 and *P. aeruginosa* PAF79 at 1000 and 800 µg/ml respectively

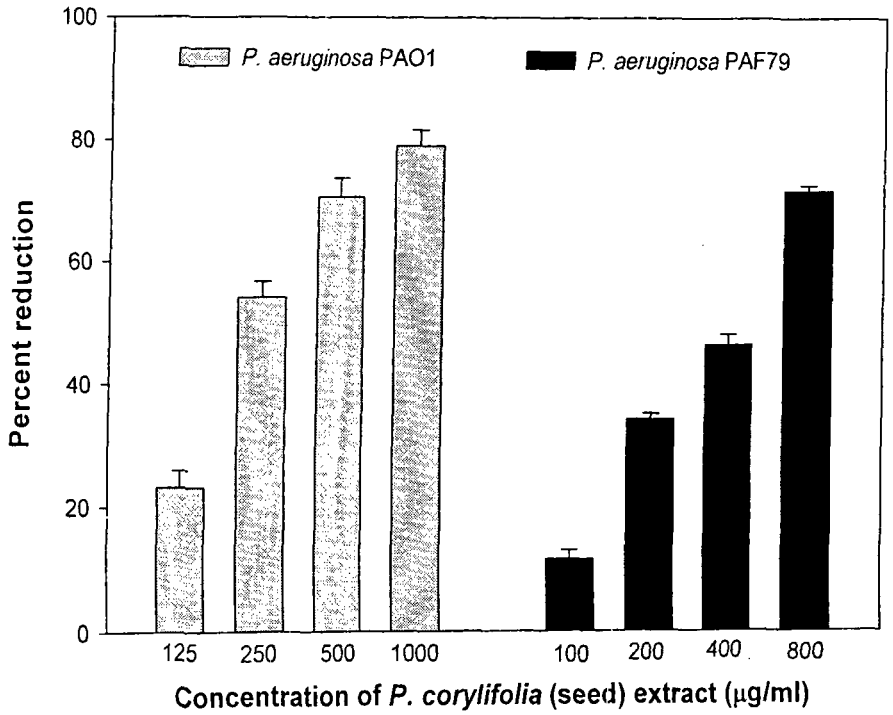


Figure 21: Effect of *P. corylifolia* (seed) extract on biofilm formation in *P. aeruginosa* PAO1 and *P. aeruginosa* PAF79 at respective sub-MICs

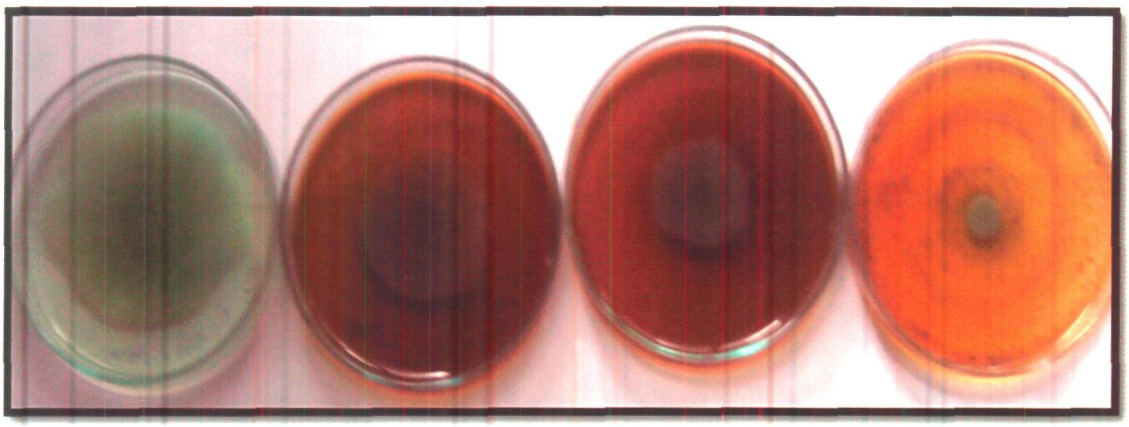


Plate 10: Inhibition of swarming motility in *P. aeruginosa* PAF79 by sub-MICs of methanol extract of *P. corylifolia* (seed), A). Untreated control; B). 200 µg/ml; C). 400 µg/ml; D). 800 µg/ml

Table 26: Effect of sub-MICs of methanolic extract of *Psoralea corylifolia* (seed) on inhibition of quorum sensing regulated virulence factors in *Aeromonas hydrophila* WAF-38

Concentration (µg/ml)	Total protease[a]	EPS production[b]	Biofilm formation[c]
Control	0.589±0.051	0.748±0.021	0.226±0.006
100	0.356±0.016 (39.5)	0.530±0.039 (29.1)	0.173±0.015 (23.4)
200	0.298±0.029 (49.4)*	0.364± 0.026(51.3)*	0.141±0.029 (37.6)
400	0.278±0.010 (52.8)*	0.29±0.013 (61.2)**	0.124±0.022 (45.6)*
800	0.203±0.004 (65.5)**	0.255±0.013 (69.9)**	0.111±0.013 (50.8)*

[a] Total protease activity is expressed as the absorbance at OD_{600}.
[b]EPS production is expressed as absorbance at OD_{480}
[c] Biofilm formation is expressed as OD_{470} after incubation with crystal violet.
The data represents mean values of three independent experiments. *, significance at $p \leq 0.05$, **, significance at $p \leq 0.005$, *** significance at $p \leq 0.001$
Values in the parentheses indicate percent reduction over control

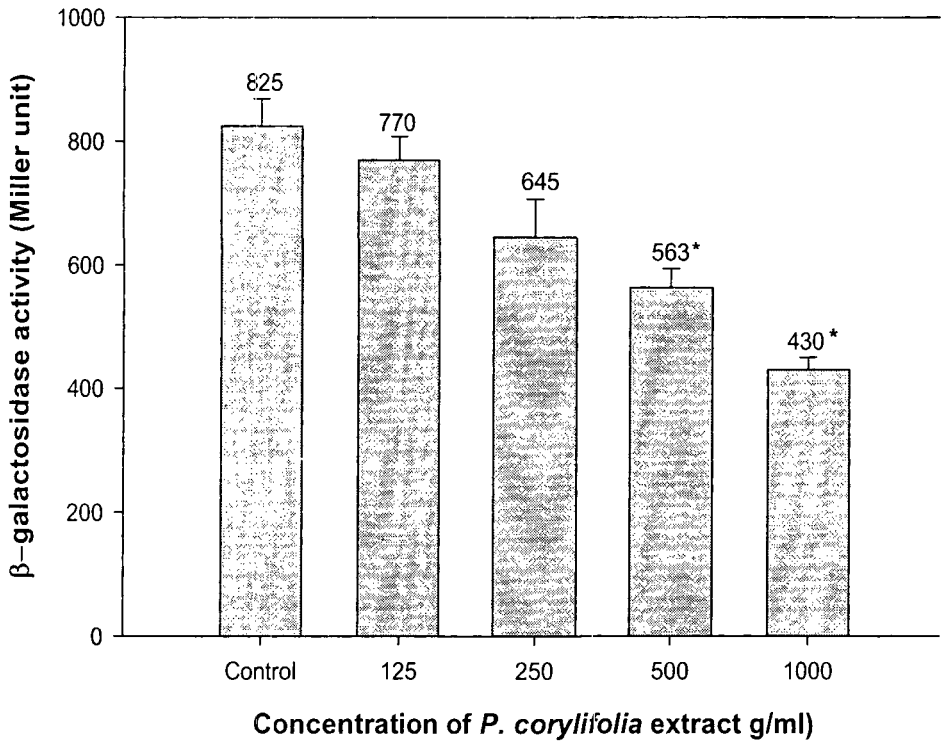


Figure 22: Effect of *P. corylifolia* (seed) extract on β-galactosidase activity in *E. coli* MG4/pKDT17.
All of the data are presented as mean ± SD. *, significance at $p \leq 0.05$

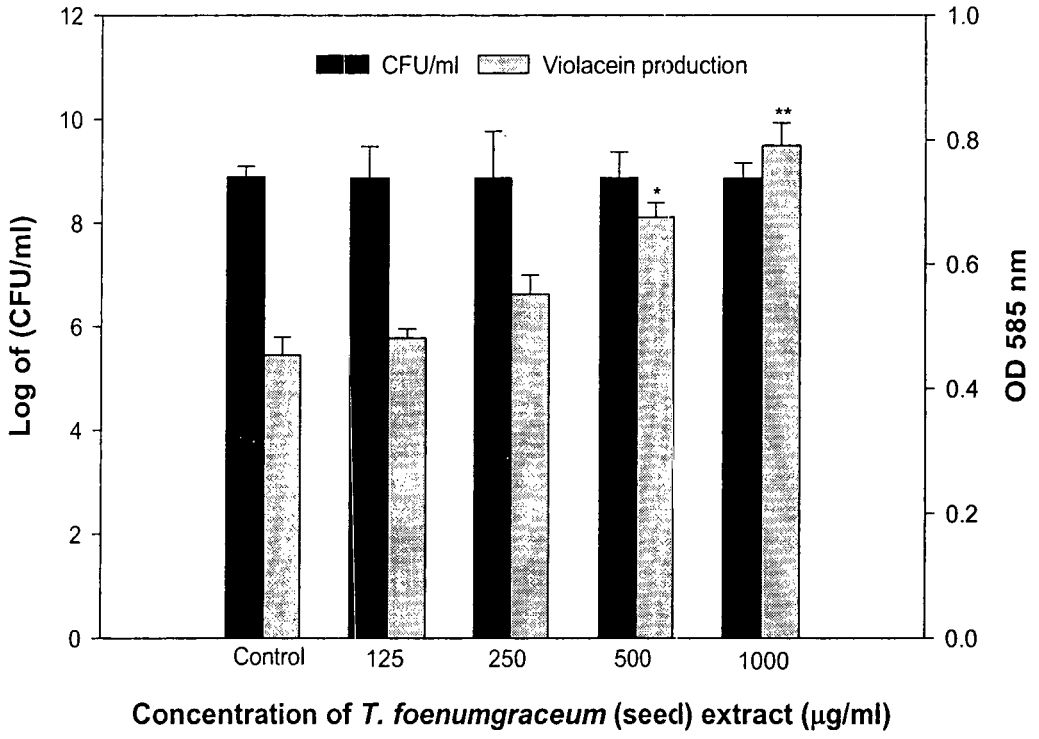


Figure 23: Quantitative assessment of violacein inhibition in CVO26 by sub-MICs of *T. foenum-graceum* (seed) extract.
All of the data are presented as mean ± SD. *, significance at $p \leq 0.05$, **, significance at $p \leq 0.005$

Table 27: Effect of sub-MICs of methanolic extract of *Trigonella foenum-graceum* (seed) on inhibition of quorum sensing regulated virulence factors in *P. aeruginosa* PAO1

Extract concentration (μg/ml)	Elastase activity[a]	Total protease[b]	Pyocyanin production[c]	Chitinase activity[d]	EPS production[e]	Swarming motility[f]	Biofilm formation[g]
Control	0.181±0.044	1.110±0.027	5.2±0.6	0.120±0.009	1.075±0.015	72±1.5	0.406±0.039
125	0.138±0.040 (23.7)	0.770±0.024 (30.6)	4.3±0.72 (17.3)	0.094±0.003 (21.6)	1.039±0.026 (03.3)	57±3.2 (20.8)	0.308±0.021 (24.1)
250	0.124±0.037 (31.4)	0.715±0.019 (35.5)	4.0±0.35 (23.0)	0.080±0.013 (33.3)	0.916±0.023 (14.7)	42±1.8 (41.6)*	0.223±0.025 (45.0)*
500	0.086±0.020 (52.4)**	0.480±0.010 (56.7)*	2.8±0.39 (46.1)*	0.069±0.013 (42.5)*	0.825±0.023 (23.2)	38±1.1 (47.2)*	0.159±0.026 (60.8)**
1000	0.070±0.025 (61.3)**	0.447±0.008 (59.7)*	2.3±0.11 (55.6)*	0.063±0.008 (47.5)*	0.575±0.016 (46.5)*	29±2.0 (59.7)**	0.127±0.041 (68.7)**

[a]Elastase activity is expressed as the absorbance at OD_{495}.
[b]Total protease activity is expressed as the absorbance at OD_{600}.
[c]Pyocyanin concentrations were expressed as micrograms of pyocyanin produced per microgram of total protein.
[d]Chitinase activity is expressed as the absorbance at OD_{570}.
[e] EPS production is expressed as absorbance at OD_{480}
[f]Swarming motility is expressed as diameter of swarm in mm
[g]Biofilm formation is expressed as OD_{470} after incubation with crystal violet
The data represents mean values of three independent experiments. *, significance at $p \leq 0.05$, **, significance at $p \leq 0.005$, *** significance at $p \leq 0.001$
Values in the parentheses indicate percent reduction over control

Table 28: Effect of sub-MICs of methanolic extract of *Trigonella foenum-graceum* (seed) on inhibition of quorum sensing regulated virulence factors in *P. aeruginosa* PAF-79

Extract concentration (μg/ml)	Elastase activity[a]	Total protease[b]	Pyocyanin production[c]	Chitinase activity[d]	EPS production[e]	Swarming motility[f]	Biofilm formation[g]
Control	0.167±0.025	1.039±0.041	3.8±0.25	0.139±0.005	0.886±0.036	48±1.5	0.331±0.027
125	0.105±0.022 (37.1)	0.564±0.024 (45.7)*	2..6±0.42 (31.5)*	0.094±0.016 (32.3)	0.414±0.025 (53.2)*	39±2 (18.7)	0.215±0.011 (35)*
250	0.085±0.021 (49.1)*	0.547±0.033 (47.3)*	1.2±0.27 (68.4)**	0.065±0.023 (53.3)*	0.357±0.019 (59.7)**	32±2 (33.3)*	0..182±0.018 (45)*
500	0.057±0.016 (65.8)*	0.499±0.003 (51.9)*	0.94±0.016 (75.2)**	0.038±0.006 (72.6)**	0.309±0.031 (65.1)**	23±0.5 (52)*	0.157±0.008 (52.5)*
1000	0.054±0.003 (67.6)*	0.468±0.010 (55)*	0.68±0.028 (82.1)***	0.018±0.004 (87)***	0.199±0.014 (77.5)**	18±3 (62.5)*	0.114±0.023 (65.5)**

[a]Elastase activity is expressed as the absorbance at OD_{495}.
[b]Total protease activity is expressed as the absorbance at OD_{600}.
[c]Pyocyanin concentrations were expressed as micrograms of pyocyanin produced per microgram of total protein.
[d]Chitinase activity is expressed as the absorbance at OD_{570}.
[e] EPS production is expressed as absorbance at OD_{480}
[f]Swarming motility is expressed as diameter of swarm in mm
[g]Biofilm formation is expressed as OD_{470} after incubation with crystal violet
The data represents mean values of three independent experiments. *, significance at $p \leq 0.05$, **, significance at $p \leq 0.005$, *** significance at $p \leq 0.001$
Values in the parentheses indicate percent reduction over control

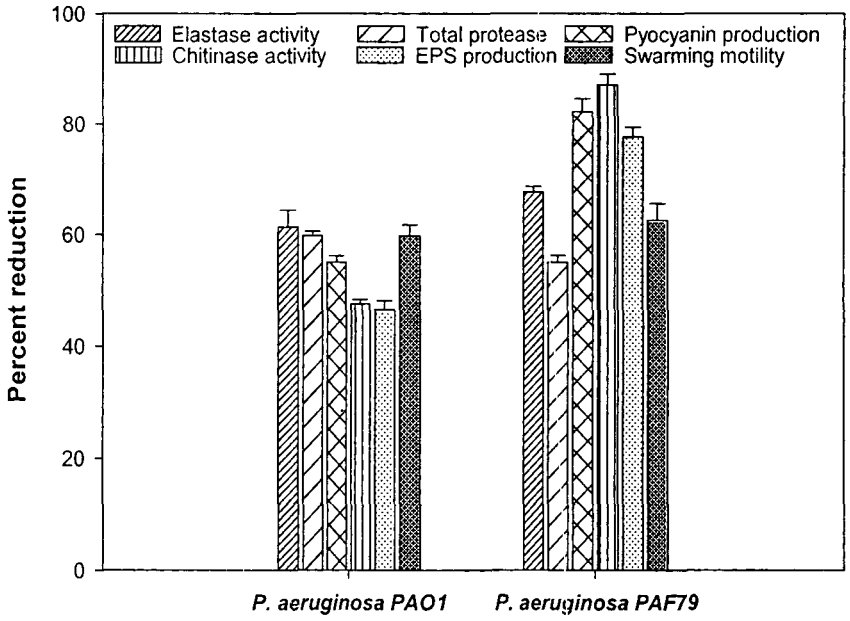


Figure 24: Effect of *T. foenum-graceum* (seed) extract on quorum sensing regulated factors in *P. aeruginosa* PAO1 and *P. aeruginosa* PAF79 at 1000 µg/ml respectively

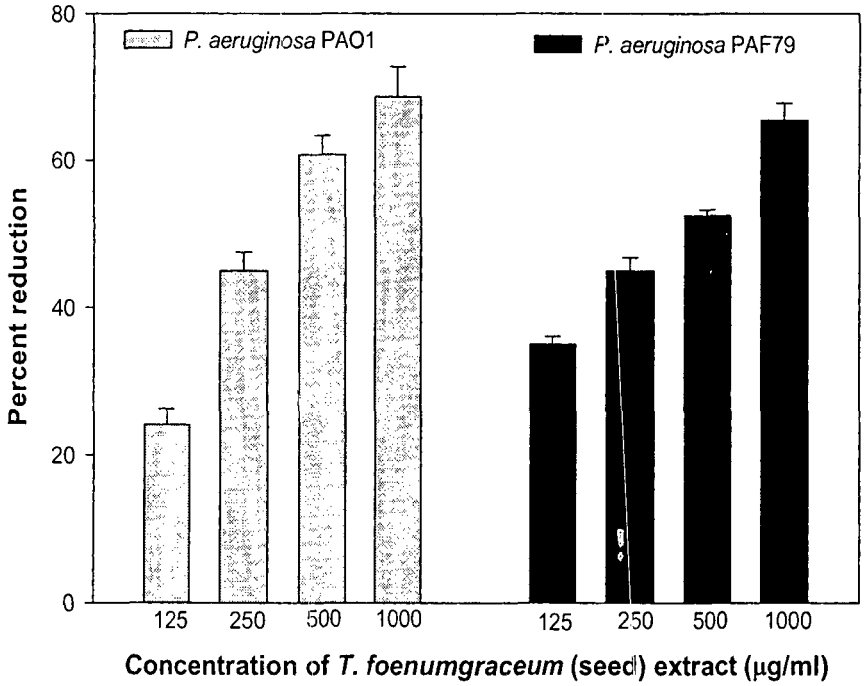


Figure 25: Effect of *T. foenum-graceum* (seed) extract on biofilm formation in *P. aeruginosa* PAO1 and *P. aeruginosa* PAF79 at respective sub-MICs

Table 29: Effect of sub-MICs of methanolic extract of *Trigonella foenum-graceum* (seed) on inhibition of quorum sensing regulated virulence factors in *Aeromonas hydrophila* WAF-38

Concentration (μg/ml)	Total protease[a]	EPS production[b]	Biofilm formation[c]
Control	0.589±0.051	0.748±0.021	0.226±0.006
100	0.520±0.025 (11.7)	0.721±0.064 (03.6)	0.126±0.011 (44.2)*
200	0.431±0.049 (26.8)	0.642± 0.035 (14.1)	0.077±0.018 (65.9)**
400	0.321±0.033 (45.5)*	0.516±0.013 (31.0)	0.068±0.004 (69.9)**
800	0.167±0.018 (71.6)**	0.401±0.021 (46.3)*	0.052±0.010 (76.9)**

[a] Total protease activity is expressed as the absorbance at OD_{600}.
[b]EPS production is expressed as absorbance at OD_{480}
[c] Biofilm formation is expressed as OD_{470} after incubation with crystal violet.
The data represents mean values of three independent experiments. *, significance at $p \leq 0.05$, **, significance at $p \leq 0.005$, *** significance at $p \leq 0.001$
Values in the parentheses indicate percent reduction over control

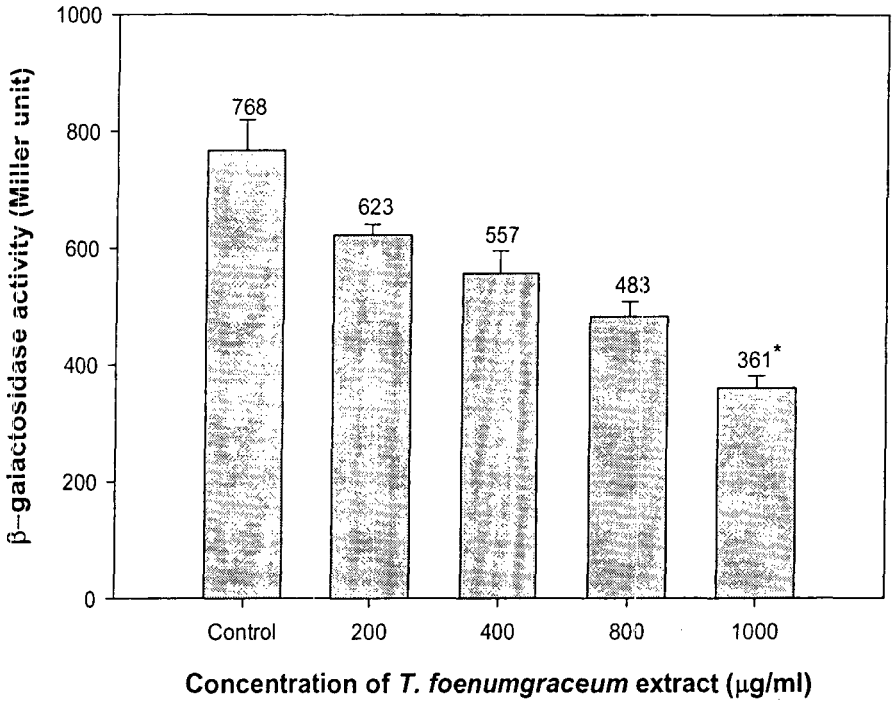


Figure 26: Effect of *T. foenum-graceum* (seed) extract on β-galactosidase activity in *E. coli* MG4/pKDT17.
All of the data are presented as mean ± SD. *, significance at $p \leq 0.05$, **, significance at $p \leq 0.005$

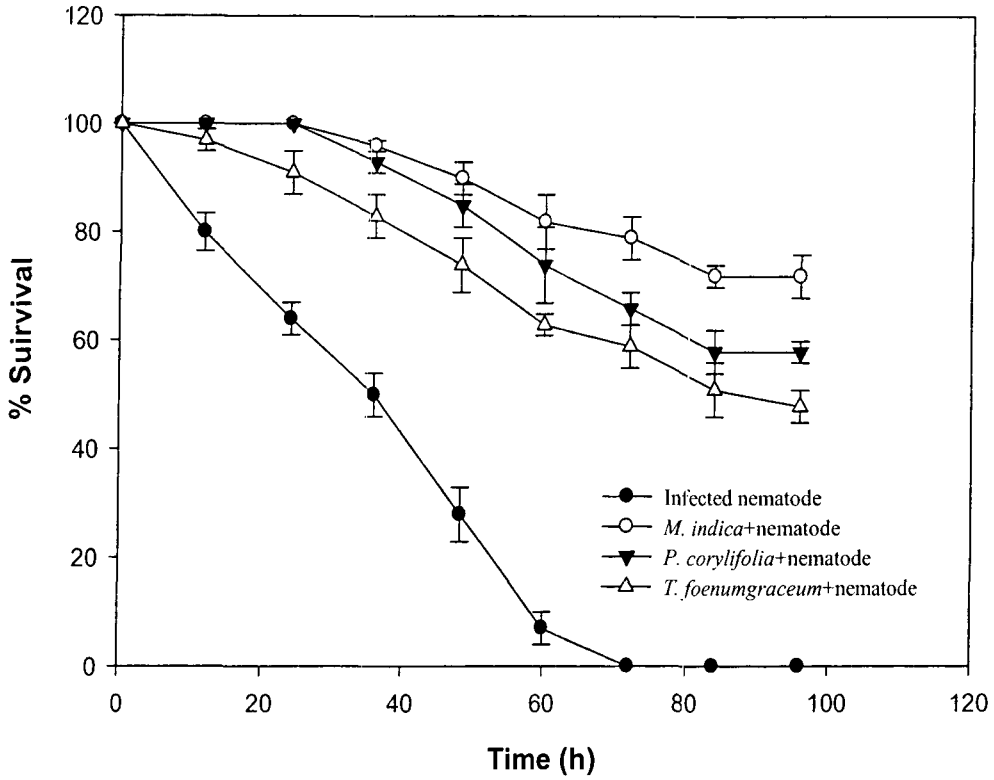


Figure 27: Anti-infection potential of sub-MICs of *M. indica* (seed) extract, *P. corylifolia* (seed) extract (1000 µg/ml) and *T. foenum-graceum* (seed) extract at respective sub-MICs (800, 1000 and 1000 µg/ml) pre-infected *C. elegans* nematode model.
Means values of triplicate independent experiments and SDs are shown.

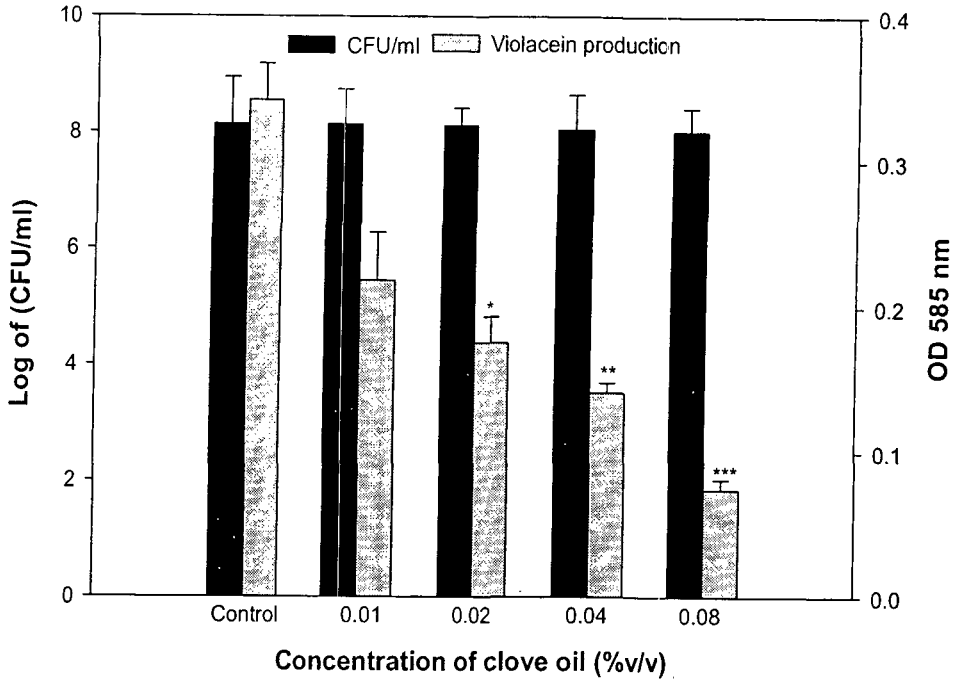


Figure 28: Quantitative assessment of violacein inhibition in CVO26 by sub-MICs of clove oil. All of the data are presented as mean ± SD. *, significance at $p \leq 0.05$, **, significance at $p \leq 0.005$, ***, significance at $p \leq 0.001$

Table 30: Effect of sub-MICs of clove oil on inhibition of quorum sensing regulated virulence factors in *P. aeruginosa* PAO1

Concentration (% v/v)	Elastase activity[a]	Total protease[b]	Pyocyanin production[c]	Chitinase activity[d]	EPS production[e]	Swarming motility[f]	Biofilm formation[g]
Control	0.132±0.018	1.169±0.034	5.91±0.47	0.158±0.013	1.278±0.02	50±2.0	0.406±0.017
0.2	0.113±0.008 (14)	0.606±0.017 (48)**	3.74±0.41 (37)	0.132±0.014 (16)	0.875±0.017 (31)***	24±1.2 (52)**	0.326±0.018 (20)*
0.4	0.088±0.012 (33)	0.457±0.016 (61)**	3.15±0.27 (47)**	0.114±0.008 (28)*	0.514±0.021 (60)***	20±0.5 (60)**	0.279±0.021 (31)*
0.8	0.067±0.014 (49)*	0.271±0.013 (76)**	2.35±0.29 (60)**	0.066±0.010 (58)*	0.448±0.012 (65)***	17±1.0 (66)***	0.203±0.019 (50)*
1.6	0.040±0.008 (69)*	0.177±0.014 (85)***	1.47±0.25 (75)**	0.031±0.003 (80)**	0.290±0.013 (77)***	10±0.5 (80)***	0.140±0.004 (65)**

[a]Elastase activity is expressed as the absorbance at OD_{495}.
[b]Total protease activity is expressed as the absorbance at OD_{600}.
[c]Pyocyanin concentrations were expressed as micrograms of pyocyanin produced per microgram of total protein.
[d]Chitinase activity is expressed as the absorbance at OD_{570}.
[e] EPS production is expressed as absorbance at OD_{480}
[f]Swarming motility is expressed as diameter of swarm in mm
[g]Biofilm formation is expressed as OD_{470} after incubation with crystal violet
The data represents mean values of three independent experiments. *, significance at $p \leq 0.05$, **, significance at $p \leq 0.005$, *** significance at $p \leq 0.001$
Values in the parentheses indicate percent reduction over control

Table 31: Effect of sub-MICs of clove oil on inhibition of quorum sensing regulated virulence factors in *P. aeruginosa* PAF-79

Concentration (% v/v)	Elastase activity[a]	Total protease[b]	Pyocyanin production[c]	Chitinase activity[d]	EPS production[e]	Swarming motility[f]	Biofilm formation[g]
Control	0.128±0.012	1.12±0.067	4.5±0.18	0.110±0.034	1.42±0.045	60±0.9	0.288±0.019
0.4	0.101±0.024 (21)	0.642±0.038 (42.6)*	3.3±0.2 (26.6)	0.100±0.030 (9)	1.01±0.05 (28.8)	46±1.4 (23.3)*	0.217±0.028 (24.6)
0.8	0.084±0.016 (34.3)	0.501±0.035 (55.2)*	1.91±0.14 (57.7)*	0.083±0.009 (24.5)	0.976±0.022 (31.2)	39±1.5 (35)*	0.101±0.030 (64.9)**
1.6	0.069±0.008 (46)	0.394±0.012 (64.8)*	1.44±0.14 (68)**	0.060±0.010 (45.4)*	0.9±0.029 (36.6)	25±0.8 (58.3)*	0.054±0.018 (81.2)**
3.2	0.040±0.009 (68.7)**	0.345±0.028 (69.1)**	0.92±0.056 (79.5)***	0.052±0.006 (52.7)*	0.641±0.031 (54.8)*	17±1 (71.6)**	0.048±0.017 (83.3)***

[a]Elastase activity is expressed as the absorbance at OD_{495}.
[b]Total protease activity is expressed as the absorbance at OD_{600}.
[c]Pyocyanin concentrations were expressed as micrograms of pyocyanin produced per microgram of total protein.
[d]Chitinase activity is expressed as the absorbance at OD_{570}.
[e] EPS production is expressed as absorbance at OD_{480}
[f]Swarming motility is expressed as diameter of swarm in mm
[g]Biofilm formation is expressed as OD_{470} after incubation with crystal violet
The data represents mean values of three independent experiments. *, significance at $p \leq 0.05$, **, significance at $p \leq 0.005$, *** significance at $p \leq 0.001$
Values in the parentheses indicate percent reduction over control

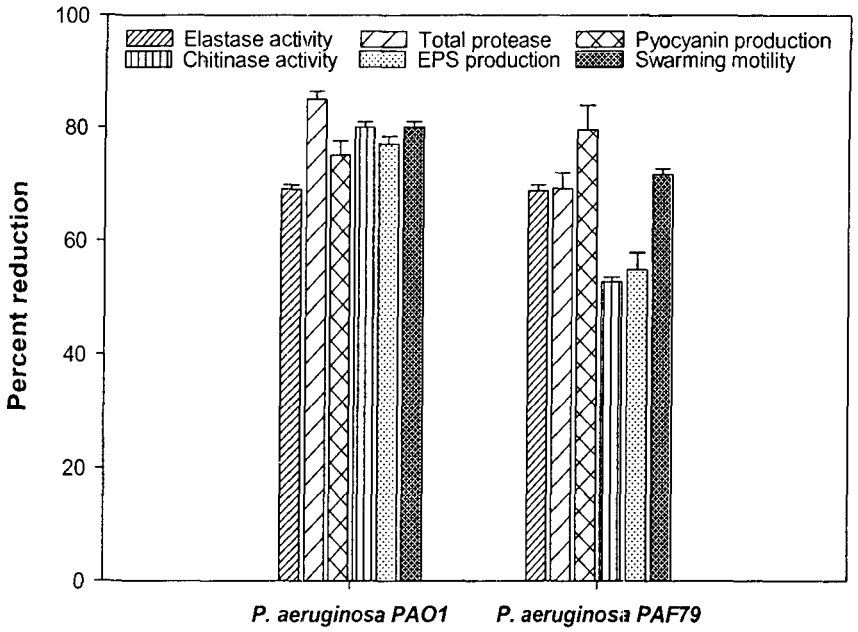


Figure 29: Effect of clove oil on quorum sensing regulated factors in *P. aeruginosa* PAO1 and *P. aeruginosa* PAF79 at 1.6 and 3.2% v/v concentration respectively

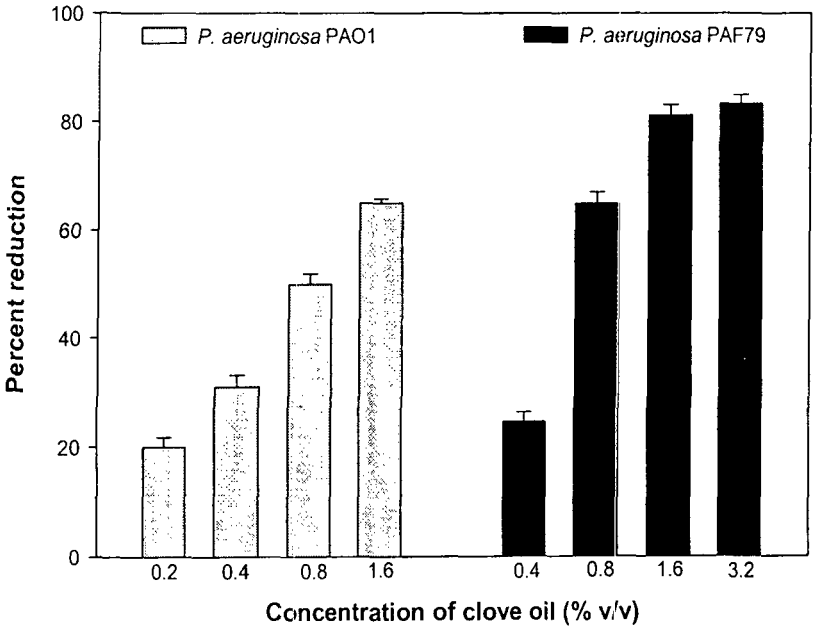


Figure 30: Effect of clove oil on biofilm formation in *P. aeruginosa* PAO1 and *P. aeruginosa* PAF79 at respective sub-MICs

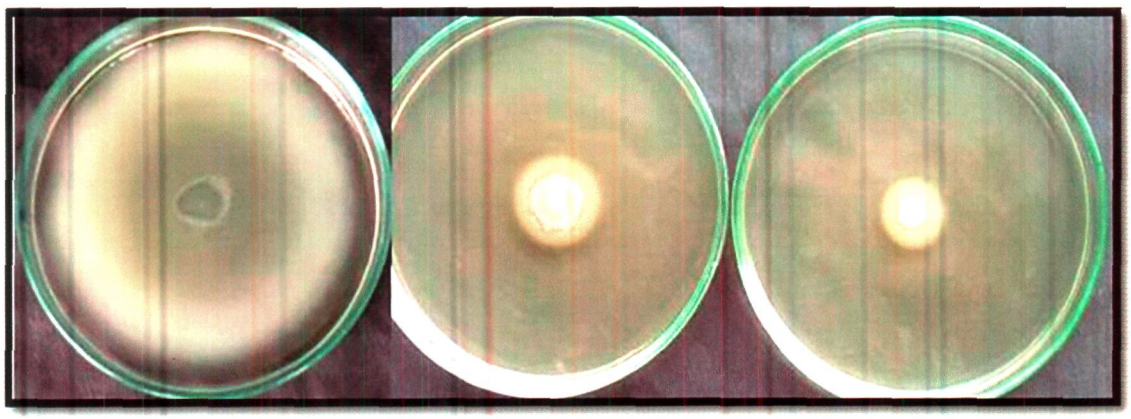


Plate 11: Inhibition of swarming motility in *P. aeruginosa* PAO1 A). Untreated control; B). peppermint oil (3.2% v/v); C). clove oil (1.6% v/v)

Table 32: Effect of sub-MICs of clove oil on inhibition of quorum sensing virulence factors in *Aeromonas hydrophila* WAF-38

Concentration (% v/v)	Total protease[a]	EPS production[b]	Biofilm formation[c]
Control	0.747±0.032	0.816±0.038	0.325±0.027
0.05	0.605±0.025 (19)	0.587±0.039 (28)	0.211±0.021 (35)*
0.1	0.515±0.028 (31)*	0.318±0.034 (61)*	0.152±0.014 (53)**
0.2	0.395±0.021 (47)*	0.261±0.022 (68)**	0.130±0.015 (60)**
0.4	0.321±0.018 (57)*	0.236±0.025 (71)**	0.110±0.008 (66)**

[a] Total protease activity is expressed as the absorbance at OD_{600}.
[b]EPS production is expressed as absorbance at OD_{480}
[c] Biofilm formation is expressed as OD_{470} after incubation with crystal violet.
The data represents mean values of three independent experiments. *, significance at $p \leq 0.05$, **, significance at $p \leq 0.005$, *** significance at $p \leq 0.001$
Values in the parentheses indicate percent reduction over control

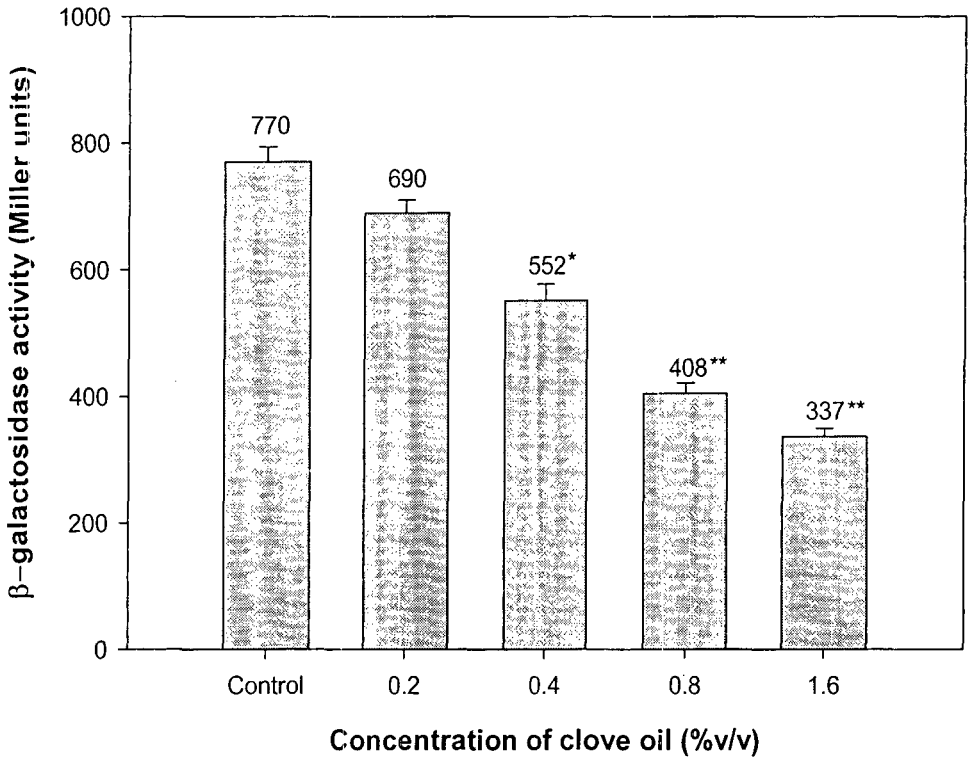


Figure 31: Effect of clove oil on β-galactosidase activity in *E. coli* MG4/pKDT17. All of the data are presented as mean ± SD. *, significance at $p \leq 0.05$, **, significance at $p \leq 0.005$

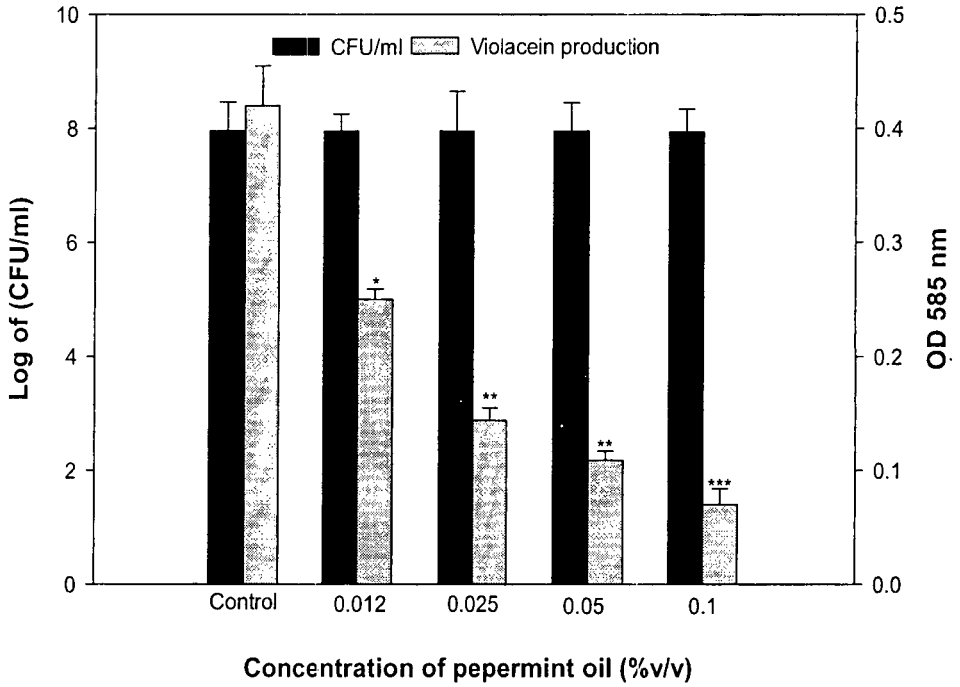


Figure 32: Quantitative assessment of violacein inhibition in CVO26 by sub-MICs of peppermint oil.
All of the data are presented as mean ± SD. *, significance at $p \leq 0.05$, **, significance at $p \leq 0.005$, ***, significance at $p \leq 0.001$

Table 33: Effect of sub-MICs of peppermint oil on inhibition of quorum sensing regulated virulence factors in *P. aeruginosa* PAO1

Concentration (% v/v)	Elastase activity[a]	Total protease[b]	Pyocyanin production[c]	Chitinase activity[d]	EPS production[e]	Swarming motility[f]	Biofilm formation[g]
Control	0.145±0.009	1.169±0.034	6.1±0.36	0.153±0.011	1.278±0.020	75±2.7	0.406±0.034
0.375	0.101±0.016 (30.3)	0.857±0.019 (26.6)	2.9±0.30 (52.4)**	0.120±0.008 (21.5)	0.767±0.013 (39.9)*	37±3.5 (50.6)**	0.232±0.021 (42.8)*
0.75	0.075±0.005 (48.2)*	0.606±0.010 (48.1)*	2.3±0.15 (62.2)**	0.090±0.013 (41.1)*	0.501±0.012 (59.2)**	29±2 (61.3)**	0.156±0.021 (61.5)*
1.5	0.050±0.008 (65.5)**	0.345±0.023 (70.4)**	1.7±0.25 (72.1)**	0.054±0.019 (64.7)*	0.449±0.010 (64.8)**	22±2 (70.6)**	0.113±0.017 (72.1)**
3	0.029±0.006 (80.0)**	0.280±0.013 (76.0)**	0.9±0.30 (85.2)***	0.032±0.006 (79.0)**	0.3±0.017 (76.52)**	14±1.5 (81.3)***	0.064±0.012 (84.2)***

[a]Elastase activity is expressed as the absorbance at OD_{495}.
[b]Total protease activity is expressed as the absorbance at OD_{600}.
[c]Pyocyanin concentrations were expressed as micrograms of pyocyanin produced per microgram of total protein.
[d]Chitinase activity is expressed as the absorbance at OD_{570}.
[e] EPS production is expressed as absorbance at OD_{480}
[f]Swarming motility is expressed as diameter of swarm in mm
[g]Biofilm formation is expressed as OD_{470} after incubation with crystal violet
The data represents mean values of three independent experiments. *, significance at $p \leq 0.05$, **, significance at $p \leq 0.005$, *** significance at $p \leq 0.001$
Values in the parentheses indicate percent reduction over control

Table 34: Effect of sub-MICs of peppermint oil on inhibition of quorum sensing regulated virulence factors in *P. aeruginosa* PAF-79

Concentration (% v/v)	Elastase activity[a]	Total protease[b]	Pyocyanin production[c]	Chitinase activity[d]	EPS production[e]	Swarming motility[f]	Biofilm formation[g]
Control	0.128±0.012	1.12±0.067	4.5±0.18	0.110±0.034	1.42±0.045	60±0.9	0.288±0.019
0.2	0.093±0.018 (27.3)	0.857±0.045 (23.4)	2.5±0.30 (44.4)**	0.087±0.030 (20.9)	0.976±0.041 (31.2)	52±0.5 (13.3)	0.188±0.009 (34.7)*
0.4	0.076±0.024 (40)*	0.748±0.021 (33.2)	1.1±0.057 (75.5)***	0.07±0.009 (36.3)*	0.891±0.02 (37.5)	49±2 (18.3)	0.123±0.013 (57.2)*
0.8	0.070±0.014 (45.3)*	0.564±0.030 (49.6)*	0.72±0.046 (84)***	0.043±0.010 (60.9)*	0.756±0.035 (46.7)*	22±0.5 (63.3)**	0.073±0.010 (74.6)**
1.6	0.025±0.002 (80)***	0.509±0.018 (54.5)*	0.57±0.022 (87.3)****	0.029±0.006 (73.6)**	0.561±0.031 (60.4)*	19±0.8 (68.3)*	0.034±0.018 (88.1)***

[a]Elastase activity is expressed as the absorbance at OD_{495}.
[b]Total protease activity is expressed as the absorbance at OD_{600}.
[c]Pyocyanin concentrations were expressed as micrograms of pyocyanin produced per microgram of total protein.
[d]Chitinase activity is expressed as the absorbance at OD_{570}.
[e] EPS production is expressed as absorbance at OD_{480}
[f]Swarming motility is expressed as diameter of swarm in mm
[g]Biofilm formation is expressed as OD_{470} after incubation with crystal violet
The data represents mean values of three independent experiments. *, significance at $p \leq 0.05$, **, significance at $p \leq 0.005$, *** significance at $p \leq 0.001$
Values in the parentheses indicate percent reduction over control

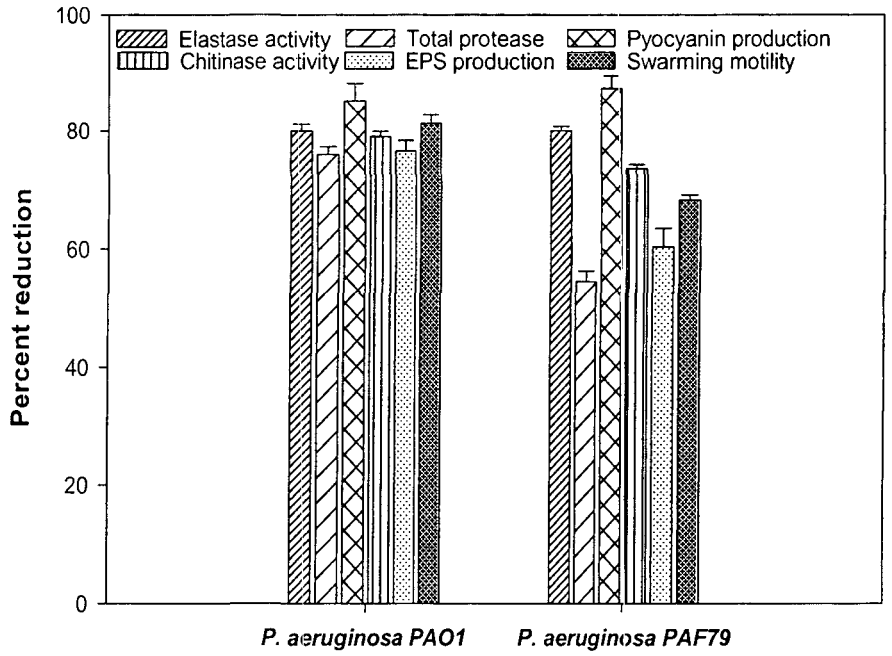


Figure 33: Effect of peppermint oil on quorum sensing regulated factors in *P. aeruginosa* PAO1 and *P. aeruginosa* PAF79 at 3 and 1.6% v/v concentration respectively

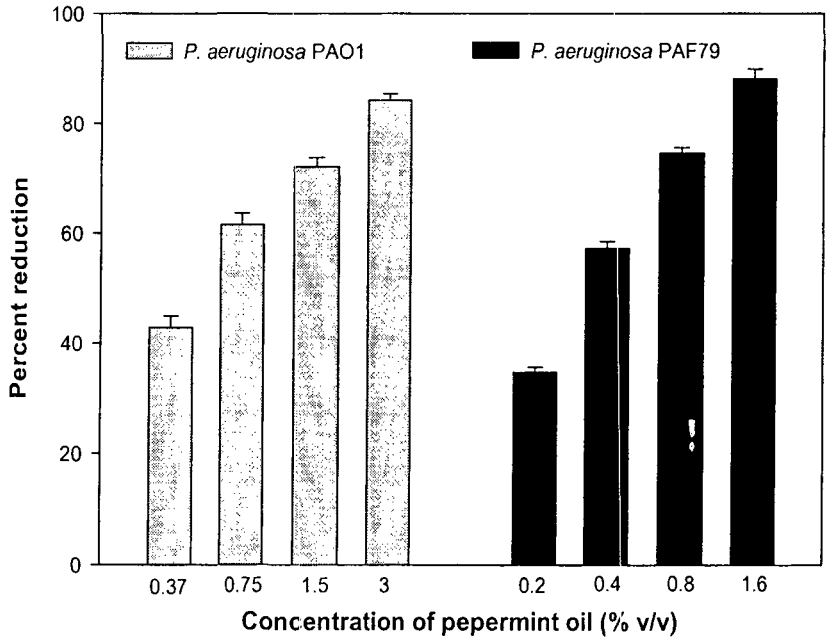


Figure 34: Effect of peppermint oil on biofilm formation in *P. aeruginosa* PAO1 and *P. aeruginosa* PAF79 at respective sub-MICs

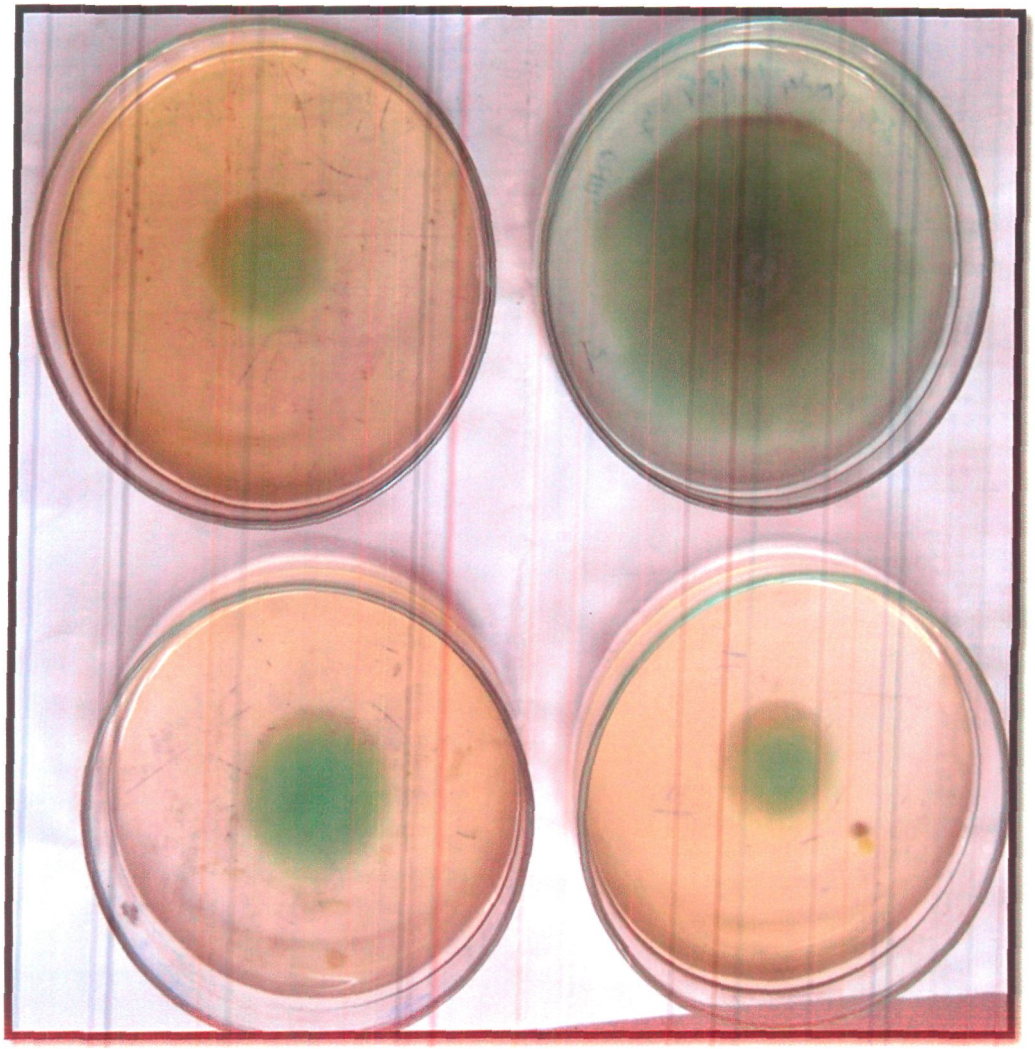


Plate 12: Inhibition of swarming motility in *P. aeruginosa* PAF79 A). Untreated control; B). Clove oil (3.2% v/v); C). Peppermint oil (1.6% v/v); D).menthol 400 µg/ml

Table 35: Effect of sub-MICs of peppermint oil on inhibition of quorum sensing regulated virulence factors in *Aeromonas hydrophila* WAF-38

Concentration (% v/v)	Total protease[a]	EPS production[b]	Biofilm formation[c]
Control	0.847±0.018	0.834±0.038	0.310±0.024
0.1	0.639±0.034 (24.5)	0.508±0.017 (39.0)	0.187±0.014 (39.6)
0.2	0.413±0.012 (51.2)*	0.330±0.011 (60.4)*	0.143±0.015 (53.8)*
0.4	0.320±0.013 (62.2)*	0.238±0.015 (71.4)**	0.090±0.021 (70.9)**
0.8	0.245±0.019 (71.0)**	0.184±0.006 (77.9)***	0.078±0.013 (74.8)**

[a] Total protease activity is expressed as the absorbance at OD_{600}.
[b]EPS production is expressed as absorbance at OD_{480}
[c] Biofilm formation is expressed as OD_{470} after incubation with crystal violet.
The data represents mean values of three independent experiments. *, significance at $p \leq 0.05$, **, significance at $p \leq 0.005$, *** significance at $p \leq 0.001$
Values in the parentheses indicate percent reduction over control

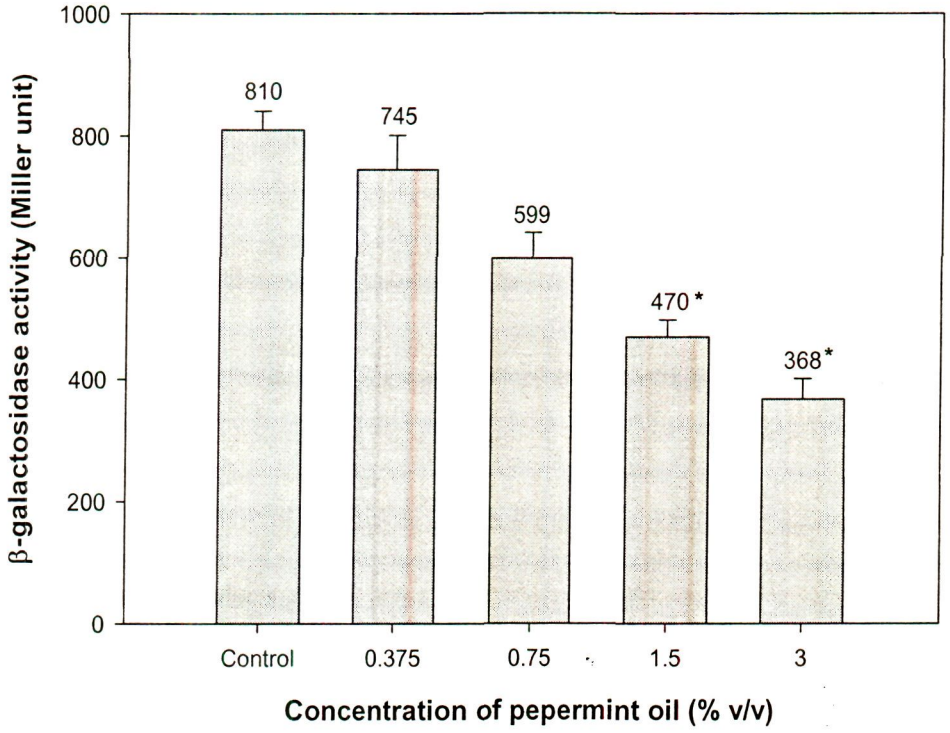


Figure 35: Effect of peppermint oil on β-galactosidase activity in *E. coli* MG4/pKDT17.
All of the data are presented as mean ± SD. *, significance at $p \leq 0.05$

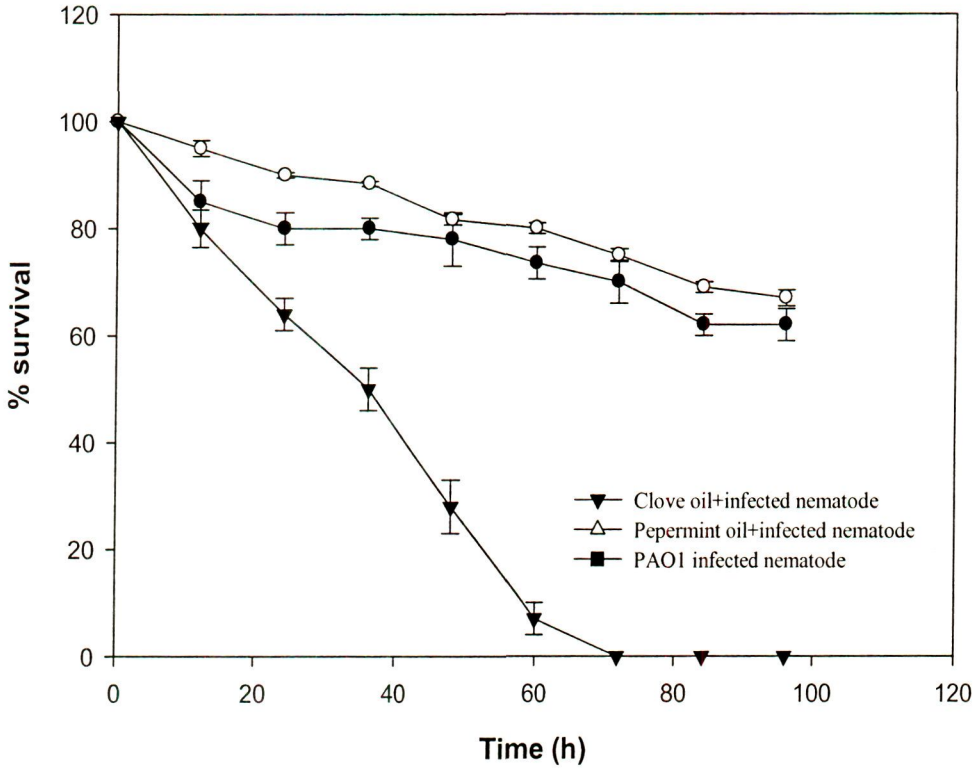


Figure 36: Anti-infection potential of of clove oil and peppermint oil (3% v/v) at respective sub-MICs (1.6 and 3% v/v) in pre-infected *C. elegans* nematode model. Means values of triplicate independent experiments and SDs are shown.

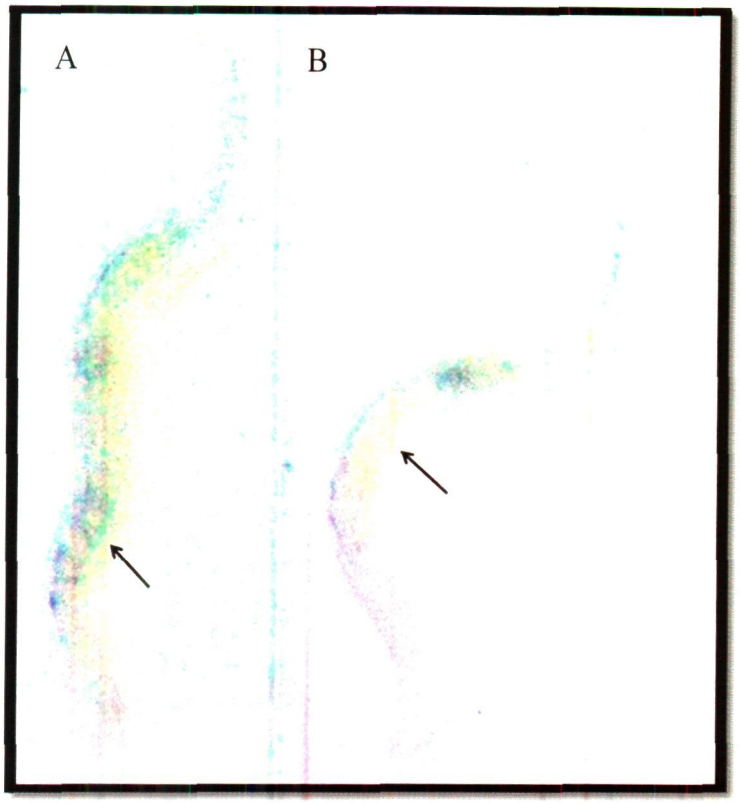


Plate 13: Anti-infective potential of peppermint oil in *C. elegans* assay. A). PAO1 infected nematode; B). Peppermint oil (3%) treated nematode .

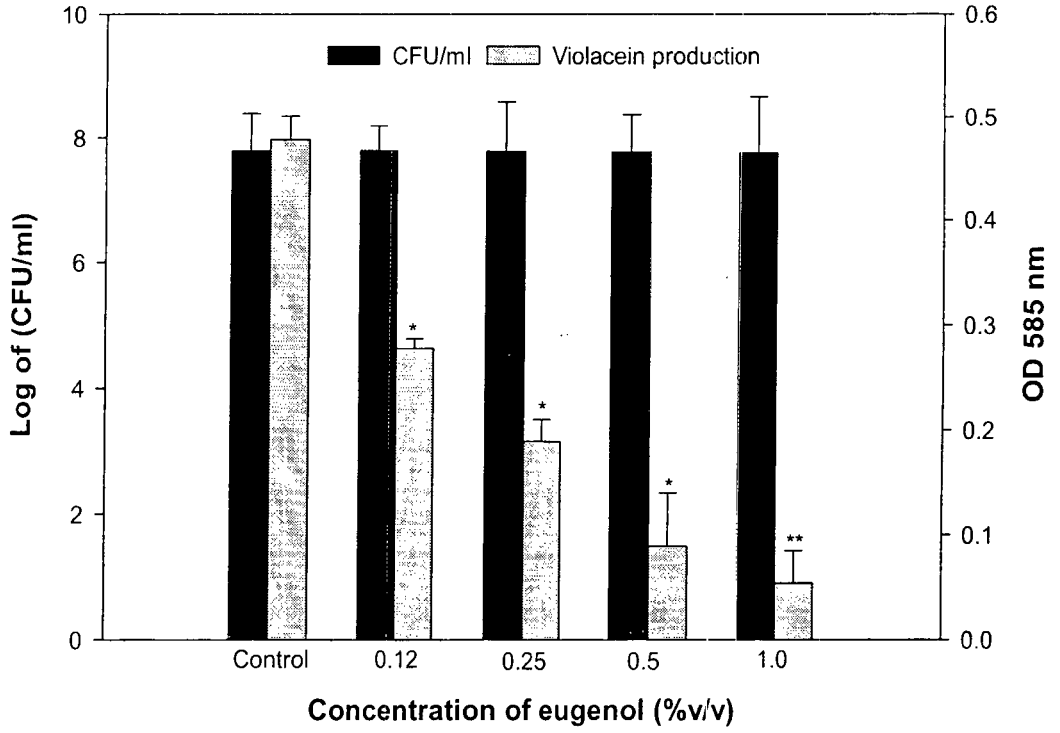


Figure 37: Quantitative assessment of violacein inhibition in CVO26 by sub-MICs of eugenol. All of the data are presented as mean ± SD. *, significance at $p \leq 0.05$, **, significance at $p \leq 0.005$

Table 36: Effect of sub-MICs of eugenol on inhibition of quorum sensing regulated virulence factors in *P. aeruginosa* PAO1

Concentration (%v/v)	Elastase activity[a]	Total protease[b]	Pyocyanin production[c]	Chitinase activity[d]	EPS production[e]	Swarming motility[f]	Biofilm formation[g]
Control	0.141±0.024	1.010±0.027	5.47±0.1	0.128±0.015	0.997±0.032	57±1.5	0.497±0.022
0.1	0.075±0.028 (47)*	0.571±0.015 (44)*	3.00±0.5 (45)*	0.091±0.024 (28.9)	0.901±0.054 (9.6)	53±3.7 (7)	0.278±0.008 (44)
0.2	0.048±0.018 (65)**	0.284±0.017 (72)**	1.8±0.035 (67)*	0.077±0.031 (39.8)*	0.752±0.042 (24.5)	52±2.0 (8.7)	0.178±0.039 (64)**
0.4	0.040±0.015 (71)**	0.177±0.022 (82)**	1.2±0.05 (78)***	0.065±0.033 (49.2)*	0.617±0.045 (38.1)	49±1.7 (14)	0.163±0.021 (67)*
0.8	0.024±0.019 (82)***	0.124±0.008 (87)***	0.8±0.045 (85)***	0.047±0.021 (63.2)*	0.507±0.029 (49.1)*	45±2.3 (21)	0.091±0.014 (81)***

[a]Elastase activity is expressed as the absorbance at OD_{495}.
[b]Total protease activity is expressed as the absorbance at OD_{600}.
[c]Pyocyanin concentrations were expressed as micrograms of pyocyanin produced per microgram of total protein.
[d]Chitinase activity is expressed as the absorbance at OD_{570}.
[e] EPS production is expressed as absorbance at OD_{480}
[f]Swarming motility is expressed as diameter of swarm in mm
[g]Biofilm formation is expressed as OD_{470} after incubation with crystal violet
The data represents mean values of three independent experiments. *, significance at $p \leq 0.05$, **, significance at $p \leq 0.005$, *** significance at $p \leq 0.001$
Values in the parentheses indicate percent reduction over control

Table 37: Effect of sub-MICs of eugenol on inhibition of quorum sensing regulated virulence factors in *P. aeruginosa* PAF-79

Concentration (%v/v)	Elastase activity[a]	Total protease[b]	Pyocyanin production[c]	Chitinase activity[d]	EPS production[e]	Swarming motility[f]	Biofilm formation[g]
Control	0.121±0.015	1.3±0.073	4.5±0.25	0.129±0.013	0.923±0.056	52±2.5	0.310±0.04
0.3	0.091±0.028 (24.7)	0.505±0.015 (61.1)**	3.1±0.8 (31.1)*	0.081±0.024 (37.2)	0. 634±0.054 (31.3)*	52±0.8 (0)	0.252±0.008 (18.7)
0.75	0.065±0.018 (46.2)*	0.371±0.017 (71.4)**	1.6±0.035 (64.4)**	0.065±0.031 (49.6)*	0. 462±0.042 (49.9)**	48±2.0 (7.6)	0.138±0.039 (55.4)**
1.5	0.040±0.015 (66.9)**	0.199±0.022 (84.6)**	1.0±0.05 (77.7)***	0.050±0.033 (61.2)*	0.402±0.045 (56.4)**	42±1.7 (19.2)	0.083±0.021 (73.2)*
3	0.030±0.019 (75.2)**	0.112±0.008 (91.3)***	0.8±0.045 (82.2)***	0.035±0.021 (72.8)**	0.278±0.029 (69.8)**	38±2.3 (26.9)	0.059±0.014 (80.9)***

[a]Elastase activity is expressed as the absorbance at OD_{495}.
[b]Total protease activity is expressed as the absorbance at OD_{600}.
[c]Pyocyanin concentrations were expressed as micrograms of pyocyanin produced per microgram of total protein.
[d]Chitinase activity is expressed as the absorbance at OD_{570}.
[e] EPS production is expressed as absorbance at OD_{480}
[f]Swarming motility is expressed as diameter of swarm in m
[g]Biofilm formation is expressed as OD_{470} after incubation with crystal violet
The data represents mean values of three independent experiments. *, significance at $p \leq 0.05$, **, significance at $p \leq 0.005$, *** significance at $p \leq 0.001$
Values in the parentheses indicate percent reduction over control

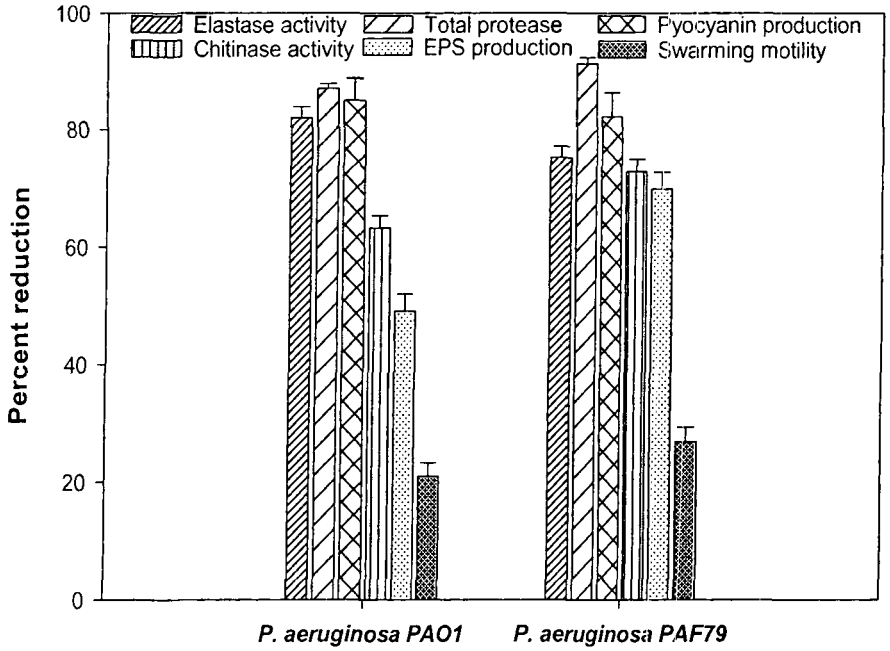


Figure 38: Effect of eugenol on quorum sensing regulated factors in *P. aeruginosa* PAO1 and *P. aeruginosa* PAF79 at 0.8 and 3% v/v concentration respectively

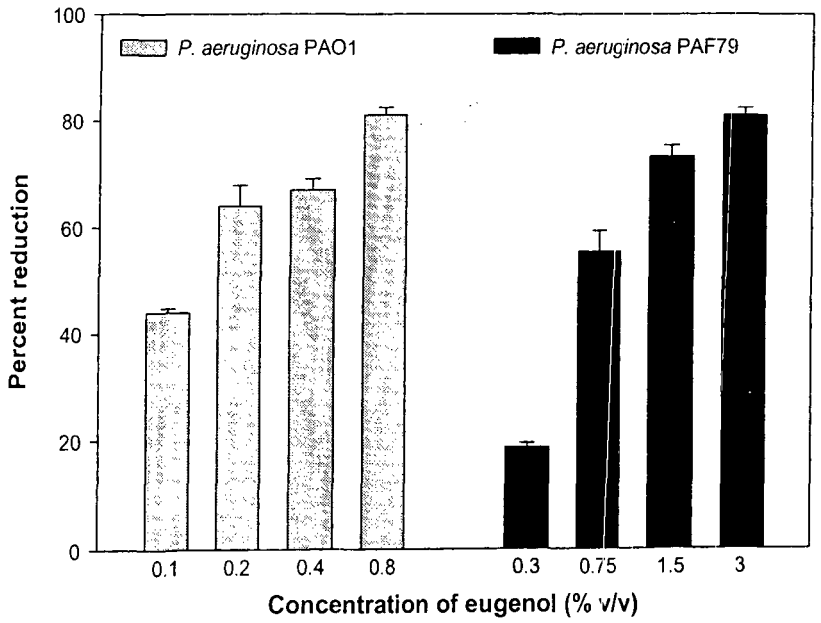


Figure 39: Effect of eugenol on biofilm formation in *P. aeruginosa* PAO1 and *P. aeruginosa* PAF79 at respective sub-MICs

Table 38: Effect of sub-MICs of eugenol on inhibition of quorum sensing regulated virulence factors in *Aeromonas hydrophila* WAF-38

Concentration (% v/v)	Total protease[a]	EPS production[b]	Biofilm formation[c]
Control	0.854±0.043	1.06±0.038	0.301±0.016
0.075	0.623±0.032 (27.0)	1.03±0.010 (02.8)	0.192±0.027 (36.2)*
0.15	0.485±0.017 (43.2)*	0.597± 0.030 (43.6)*	0.094±0.024 (68.7)**
0.3	0.379±0.023 (55.6)*	0..461±0.019 (56.5)*	0.059±0.015 (80.3)**
1.5	0.299±0.028 (64.9)*	0.319±0.021 (69.9)**	0.048±0.009 (84.0)***

[a] Total protease activity is expressed as the absorbance at OD_{600}.
[b]EPS production is expressed as absorbance at OD_{480}
[c] Biofilm formation is expressed as OD_{470} after incubation with crystal violet.
The data represents mean values of three independent experiments. *, significance at $p \leq 0.05$, **, significance at $p \leq 0.005$, *** significance at $p \leq 0.001$
Values in the parentheses indicate percent reduction over control

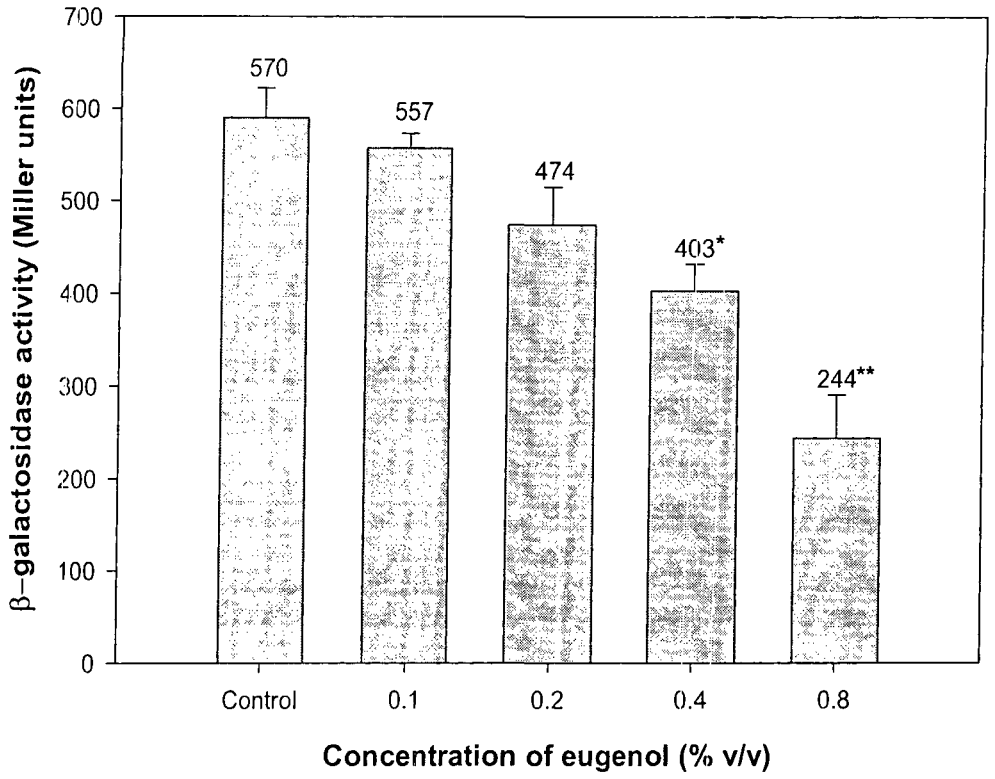


Figure 40: Effect of eugenol on β-galactosidase activity in *E. coli* MG4/pKDT17. All of the data are presented as mean ± SD. *, significance at $p \leq 0.05$, **, significance at $p \leq 0.005$

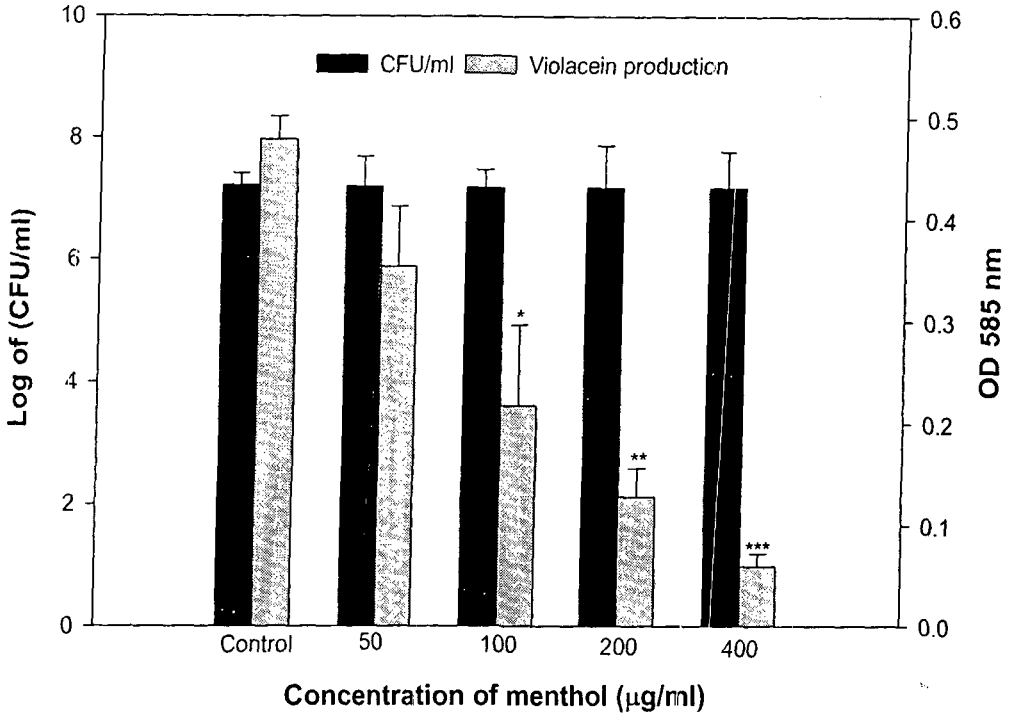


Figure 41: Quantitative assessment of violacein inhibition in CVO26 by sub-MICs of menthol. All of the data are presented as mean ± SD. *, significance at $p \leq 0.05$, **, significance at $p \leq 0.005$, ***, significance at $p \leq 0.001$

Table 39: Effect of sub-MICs of menthol on inhibition of quorum sensing regulated virulence factors in *P. aeruginosa* PAO1

Concentration (µg/ml)	Elastase activity[a]	Total protease[b]	Pyocyanin production[c]	Chitinase activity[d]	EPS production[e]	Swarming motility[f]	Biofilm formation[g]
Control	0.141±0.024	1.010±0.027	5.47±0.1	0.128±0.015	0.997±0.032	55±2.7	0.677±0.050
100	0.092±0.015 (34.7)	0.661±0.033 (34.5)	2.2±0.43 (59.7)*	0.109±0.015 (14.8)	0.612±0.038 (38.6)	41±3.5 (25)	0.577±0.022 (14.7)
200	0.062±0.019 (56)**	0.351±0.029 (65.2)**	2.0±0.1 (63.4)*	0.084±0.022 (34.3)*	0.581±0.016 (41.7)	27±4.0 (51)*	0.384±0.041 (43.2)*
400	0.034±0.012 (75.8)***	0.199±0.019 (80.2)***	1.4±0.32 (74.4)**	0.071±0.010 (44.5)*	0.515±0.014 (48.3)*	18±2.0 (67)**	0.311±0.035 (54)**
800	0.030±0.009 (78.7)**	0.159±0.019 (84.2)***	0.9±0.038 (83.5)***	0.058±0.014 (54.6)*	0.421±0.009 (57.7)*	12±3.5 (78)**	0.207±0.009 (69.4)**

[a]Elastase activity is expressed as the absorbance at OD_{495}.
[b]Total protease activity is expressed as the absorbance at OD_{600}.
[c]Pyocyanin concentrations were expressed as micrograms of pyocyanin produced per microgram of total protein.
[d]Chitinase activity is expressed as the absorbance at OD_{570}.
[e] EPS production is expressed as absorbance at OD_{480}
[f]Swarming motility is expressed as diameter of swarm in mm
[g]Biofilm formation is expressed as OD_{470} after incubation with crystal violet
The data represents mean values of three independent experiments. *, significance at $p \leq 0.05$, **, significance at $p \leq 0.005$, *** significance at $p \leq 0.001$
Values in the parentheses indicate percent reduction over control

Table 40: Effect of sub-MICs of menthol on inhibition of quorum sensing regulated virulence factors in *P. aeruginosa* PAF-79

Concentration (µg/ml)	Elastase activity[a]	Total protease[b]	Pyocyanin production[c]	Chitinase activity[d]	EPS production[e]	Swarming motility[f]	Biofilm formation[g]
Control	0.121±0.015	1.3±0.073	4.5±0.25	0.129±0.013	0.923±0.056	52±2.5	0.310±0.04
50	0.067±0.009 (44.6)	0.878±0.056 (32.4)	2.2±0.36 (51.1)*	0.105±0.016 (18.6)	0.777±0.035 (15.8)	44±1.8 (15.3)	0.199±0.034 (35.8)
100	0.048±0.021 (60.3)*	0.687±0.033 (47.1)*	1.6±0.18 (64.4)*	0.086±0.023 (33.3)	0.698±0.044 (24.3)	35±0.6 (32.6)	0..113±0.018 (63.5)*
200	0.038±0.016 (68.5)*	0.593±0.014 (54.3)*	0.9±0.1 (80.0)**	0.080±0.006 (37.9)	0.501±0.024 (45.7)*	26±1.5 (50.0)*	0.073±0.031 (76.4)**
400	0.023±0.003 (80.9)**	0.412±0.014 (68.3)**	0.54±0.15 (88.0)***	0.063±0.004 (51.1)*	0.316±0.027 (65.7)*	16±1 (69.2)**	0.070±0.026 (77.4)**

[a]Elastase activity is expressed as the absorbance at OD_{495}.
[b]Total protease activity is expressed as the absorbance at OD_{600}.
[c]Pyocyanin concentrations were expressed as micrograms of pyocyanin produced per microgram of total protein.
[d]Chitinase activity is expressed as the absorbance at OD_{570}.
[e] EPS production is expressed as absorbance at OD_{480}
[f]Swarming motility is expressed as diameter of swarm in mm
[g]Biofilm formation is expressed as OD_{470} after incubation with crystal violet
The data represents mean values of three independent experiments. *, significance at $p \leq 0.05$, **, significance at $p \leq 0.005$, *** significance at $p \leq 0.001$
Values in the parentheses indicate percent reduction over control

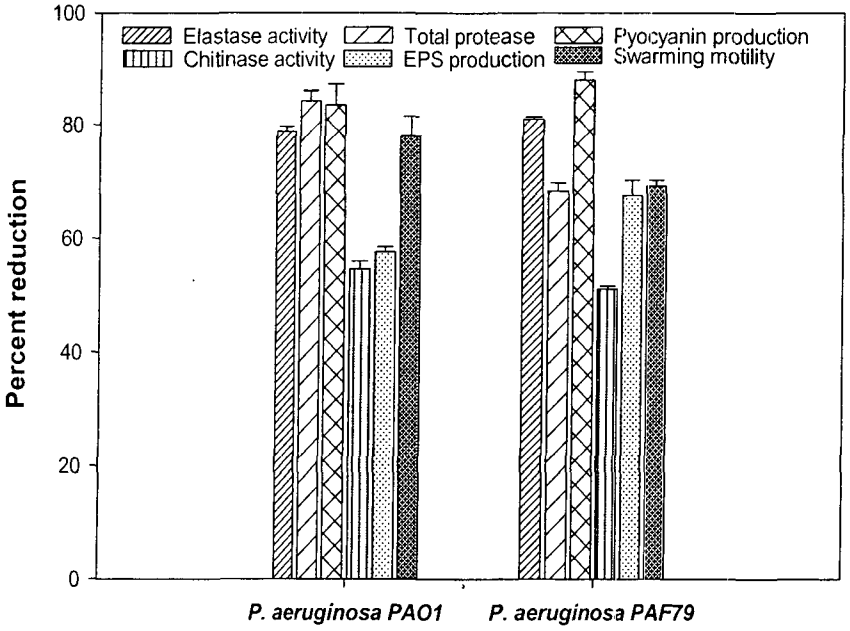


Figure 42: Effect of menthol on quorum sensing regulated factors in *P. aeruginosa* PAO1 and *P. aeruginosa* PAF79 at 800 and 400 µg/ml respectively

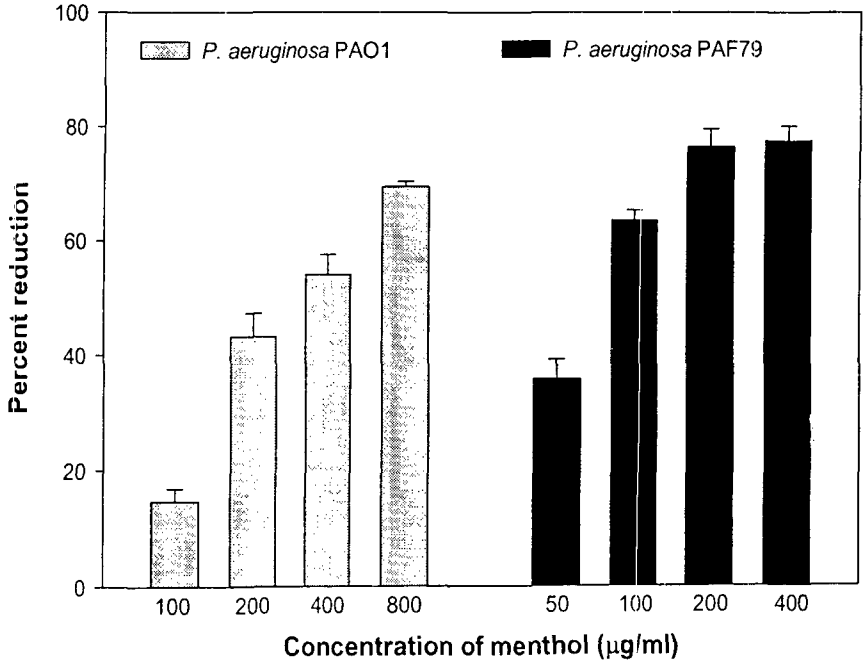


Figure 43: Effect of menthol on biofilm formation in *P. aeruginosa* PAO1 and *P. aeruginosa* PAF79 at respective sub-MICs

Table 41: Effect of sub-MICs of menthol on inhibition of quorum sensing regulated virulence factors in *Aeromonas hydrophila* WAF-38

Concentration (µg/ml)	Total protease[a]	EPS production[b]	Biofilm formation[c]
Control	0.854±0.043	1.06±0.038	0.301±0.016
25	0.754±0.024 (11.7)	0.698±0.024 (34.1)	0.217±0.031 (27.9)
50	0.636±0.023 (25.5)	0.442± 0.010 (58.3)*	0.166±0.012 (44.8)*
100	0.512±0.034 (40.0)	0..369±0.026 (65.1)*	0.082±0.007 (72.7)**
200	0.405±0.02 (52.5)*	0.353±0.018 (66.6)*	0.06±0.003 (80.0)***

[a] Total protease activity is expressed as the absorbance at OD_{600}.
[b]EPS production is expressed as absorbance at OD_{480}
[c] Biofilm formation is expressed as OD_{470} after incubation with crystal violet.
The data represents mean values of three independent experiments. *, significance at $p \leq 0.05$, **, significance at $p \leq 0.005$, *** significance at $p \leq 0.001$
Values in the parentheses indicate percent reduction over control

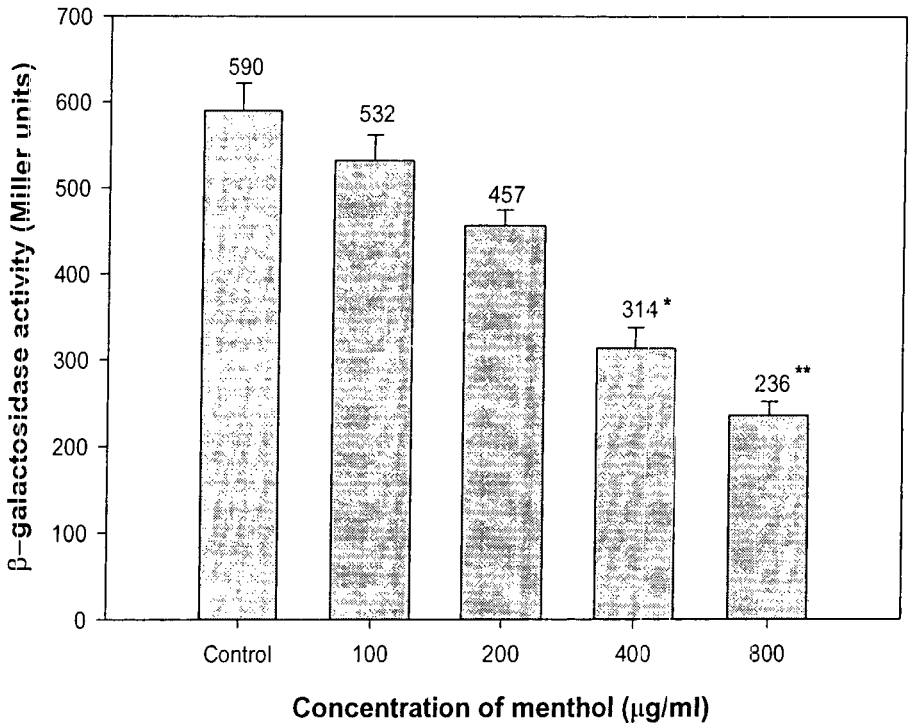


Figure 44: Effect of menthol on β-galactosidase activity in *E. coli* MG4/pKDT17. All of the data are presented as mean ± SD. *, significance at $p \leq 0.05$, **, significance at $p \leq 0.005$

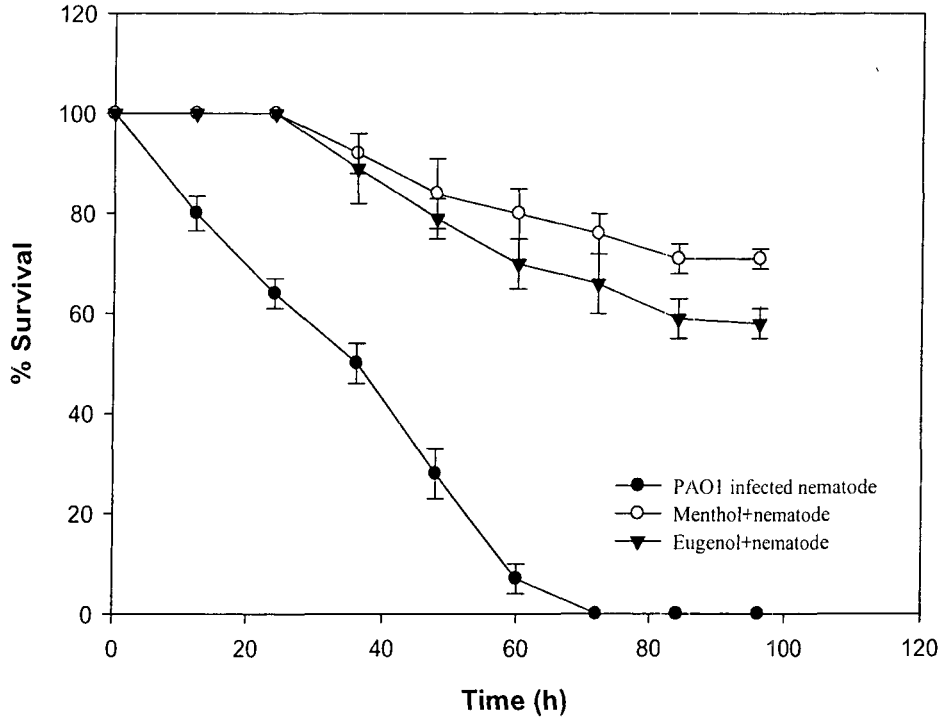


Figure 45: Anti-infection potential of eugenol and menthol at respective sub-MICs (0.5% v/v and 800 µg/ml) in pre-infected *C. elegans* nematode model.
Means values of triplicate independent experiments and SDs are shown.

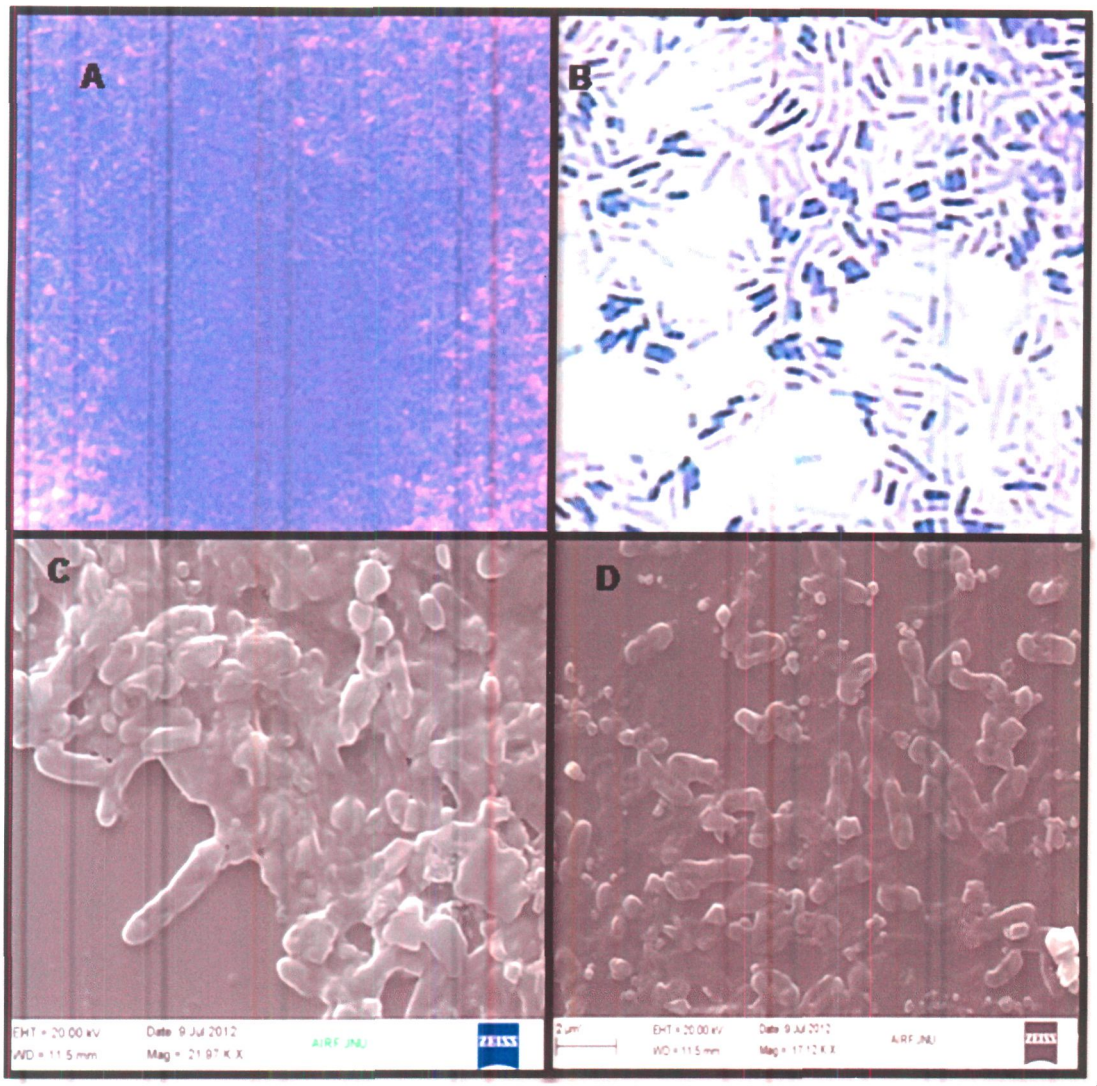


Figure 46: Microscopic images of *P. aeruginosa* PAO1 biofilm in the presence and absence of sub-MICs of clove oil. (A). Light microscopic image of untreated control, (B). treated with 1.6% (v/v) clove oil, (C). Scanning electron microscopic image of untreated control, (D). treated with 1.6% (v/v) clove oil.

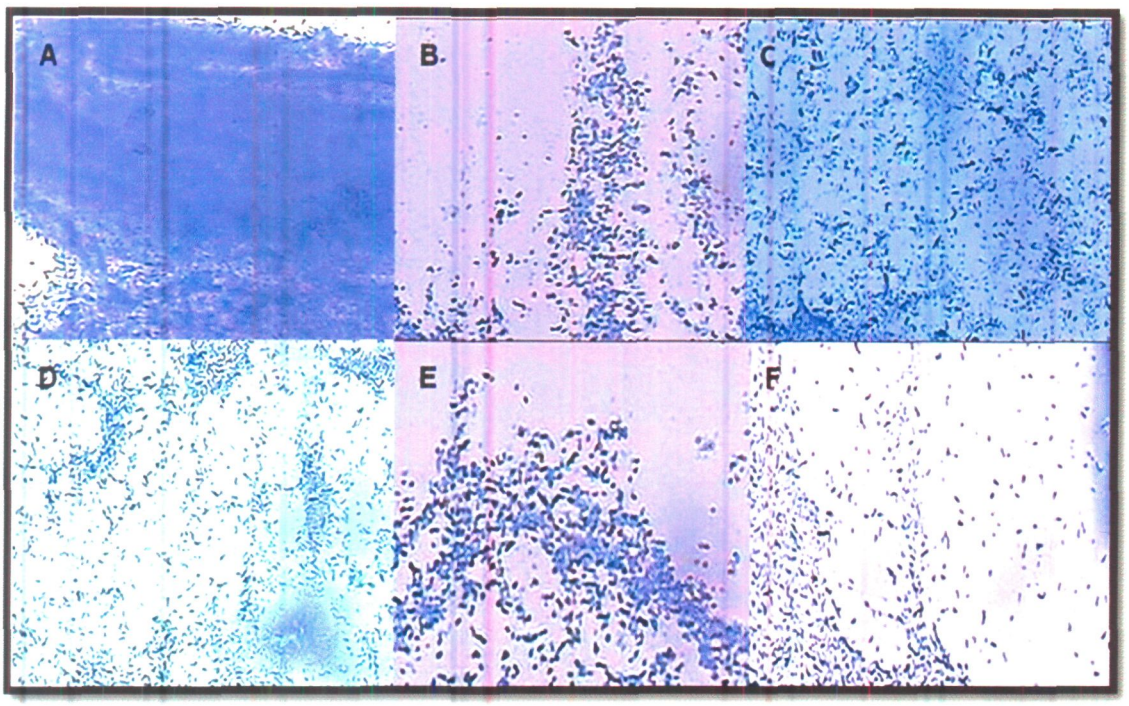


Figure 47: Inhibition of biofilm of *Pseudomonas aeruginosa* PA01 at Sub-MICs of under light microscopy A). control; B). *M. indic***a** (800 µg/ml); C). *P. corylifolia* (1000 µg/ml); D). *T. foenum-graceum* (1000 µg/ml); E). peppermint oil (3.2% v/v); F). menthol (800 µg/ml).

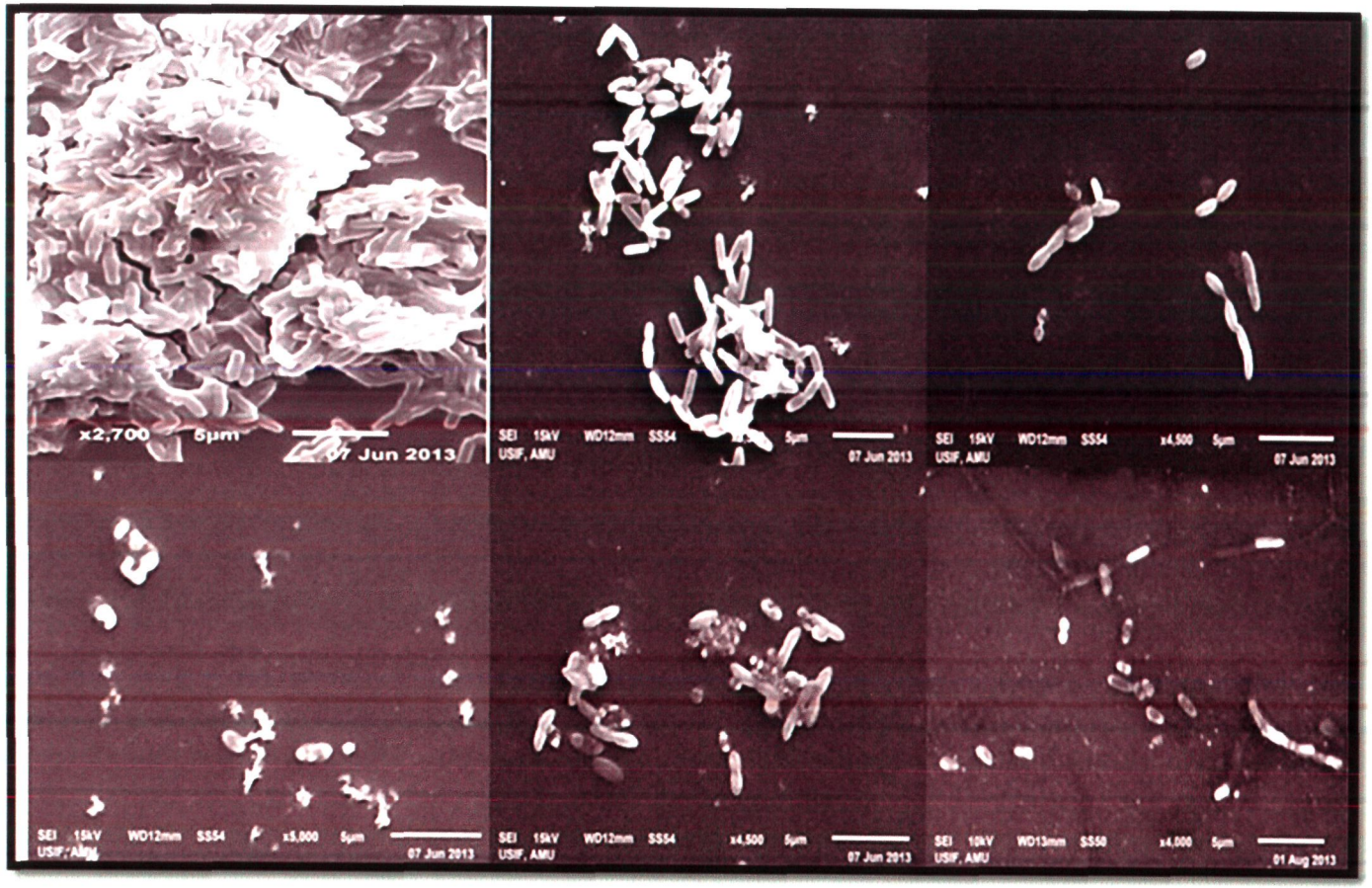


Figure 48: Scanning electron microscopic (SEM) images for inhibition of biofilm of *Pseudomonas aeruginosa* PA01 at Sub-MICs A). control; B). *M. indica* (800 μg/ml); C). *P. corylifolia* (1000 μg/ml); D). *T. foenum-graceum* (1000 μg/ml); E). peppermint oil (3.2% v/v); F). menthol (800 μg/ml).

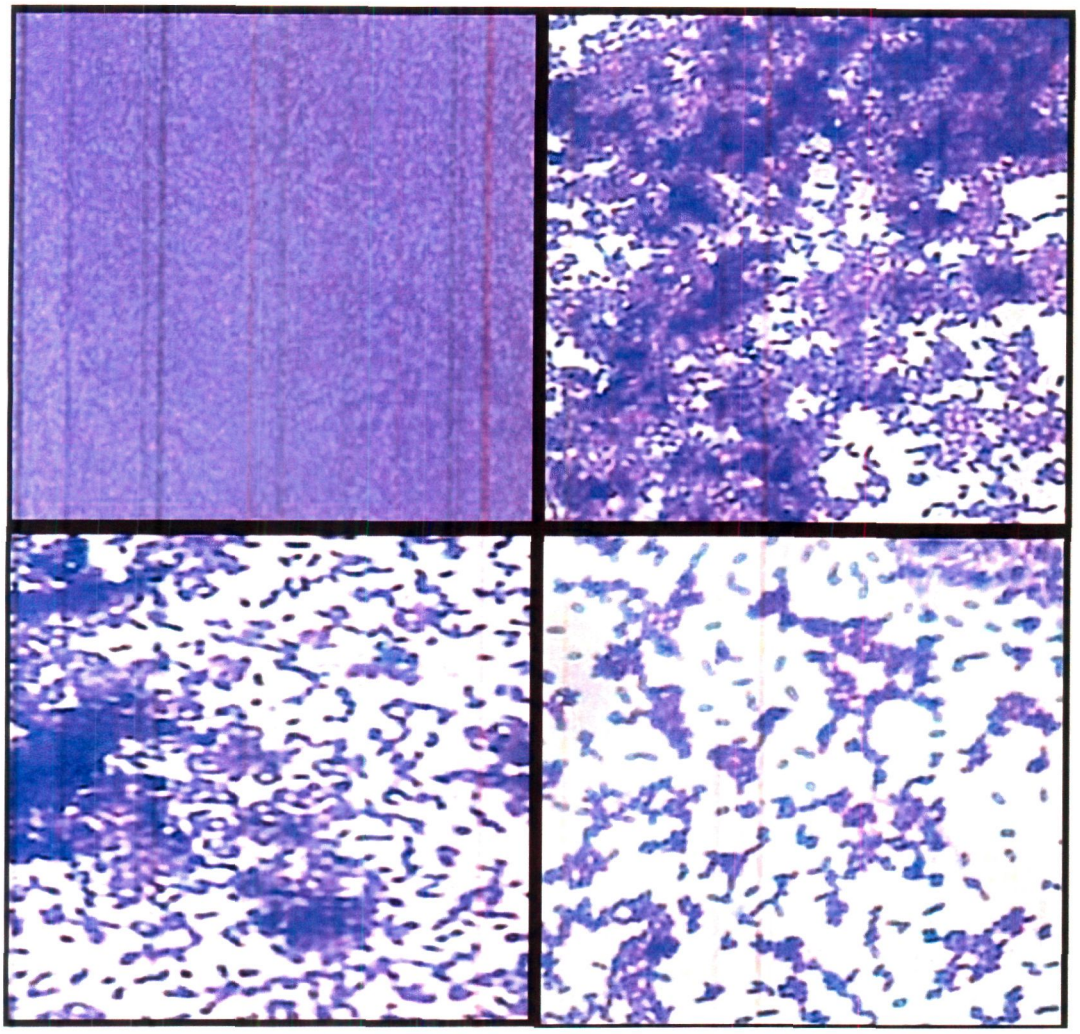


Figure 49: Light microscopiy images of *Aeromonas hydrophila* WAF-38 biofilm in the presence and absence of sub-MICs of clove oil. A). untreated control, B). treated with 0.1% (v/v) clove oil; C). treated with 0.2% (v/v) clove oil ; D). treated with 0.4% (v/v) clove oil. The cells were stained with crystal violet.

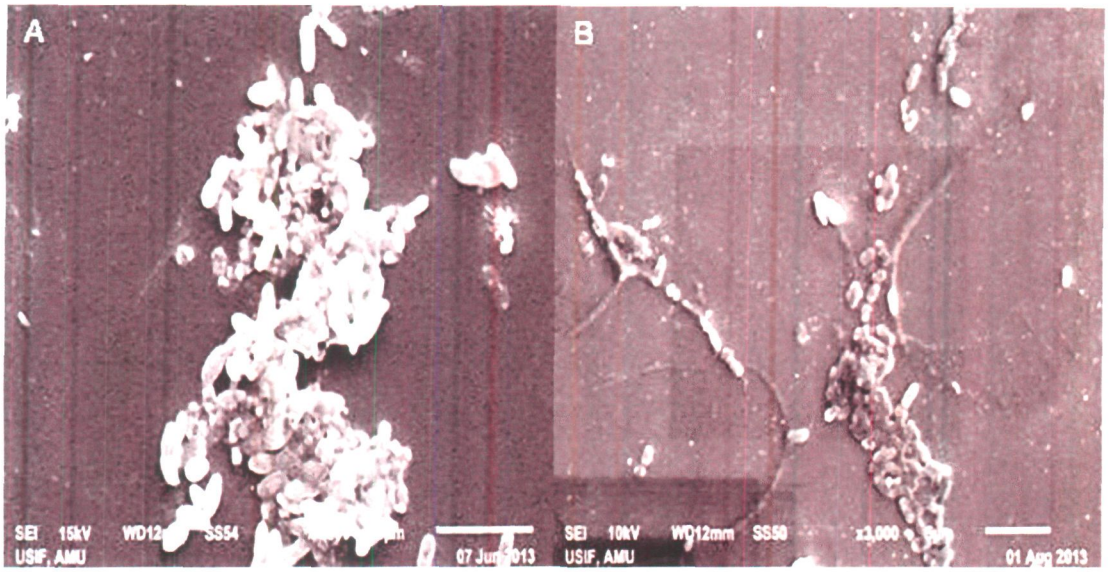


Figure 50: Scanning electron microscopic (SEM) images for inhibition of biofilm of *Aeromonas hydrophila* WAF38 at Sub-MICs A). control; B). clove oil (0.4% v/v).

Table 42a: Major groups of phytocompounds of different fractions of *Mangifera indica* (seed) extract

Name of the fraction	Alkaloids	Flavanoids	Glycosides	Phenols	Tanins
Petroleum ether	+	-	+	-	+
Benzene	-	-	+	-	+
Ethyl acetate	-	-	+	+	+
Acetone	-	-	+	+	+(Galloepitanin)
Methanol	+	+	+	+	+(Galloepitanin)

Table 42b: Major groups of phytocompounds of different fractions of *Psoralea corylifolia* (seed) extract

Name of the fraction	Alkaloids	Flavanoids	Glycosides	Phenols	Tanins
Petroleum ether	-	-	-	-	+
Benzene	-	-	-	-	+
Ethyl acetate	+	-	-	-	+(Galloepitanins)
Acetone	+	-	-	+	+(Galloepitanins)
Methanol	+	+	+	+	+(Galloepitanins)

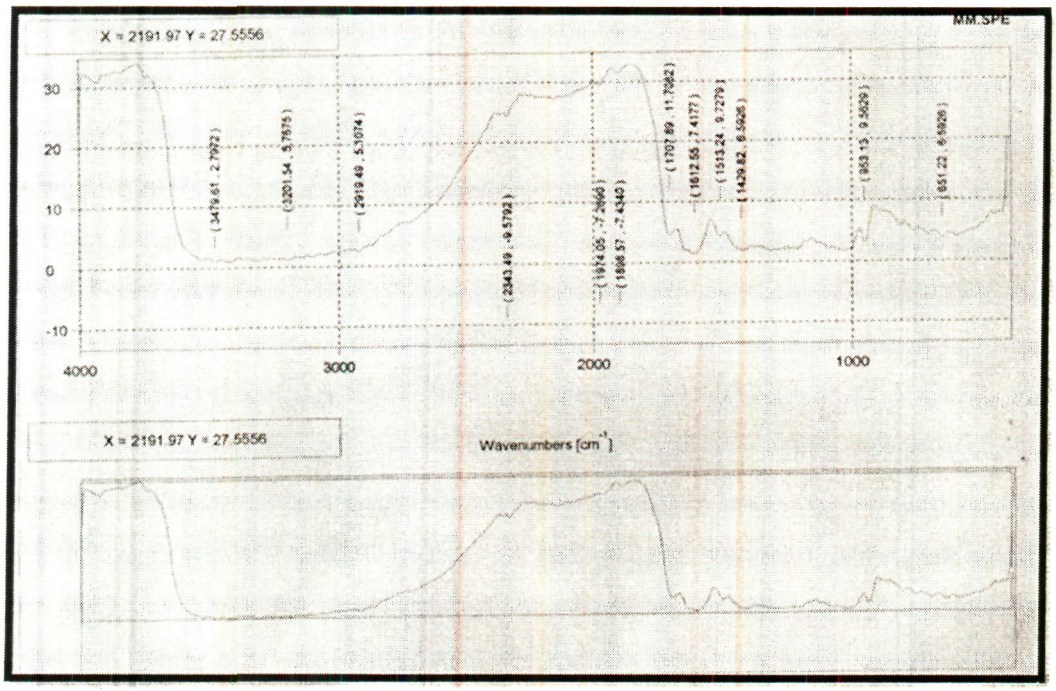
Table 42c: Major groups of phytocompounds of different fractions of *Trigonella foenumgraecum* (seed) extract

Name of the fraction	Alkaloids	Flavanoids	Glycosides	Phenols	Tanins
Petroleum ether	-	-	+	-	+
Benzene	-	-	-	-	-
Ethyl acetate	+	+	+	-	+
Acetone	+	-	+	+	+(Galloepitanins)
Methanol	+	+	+	+	-

Table 43: Total phenolics content of medicinal plants expressed as gallic acid equivalents (mg/g) by Folin's Coicalteu method

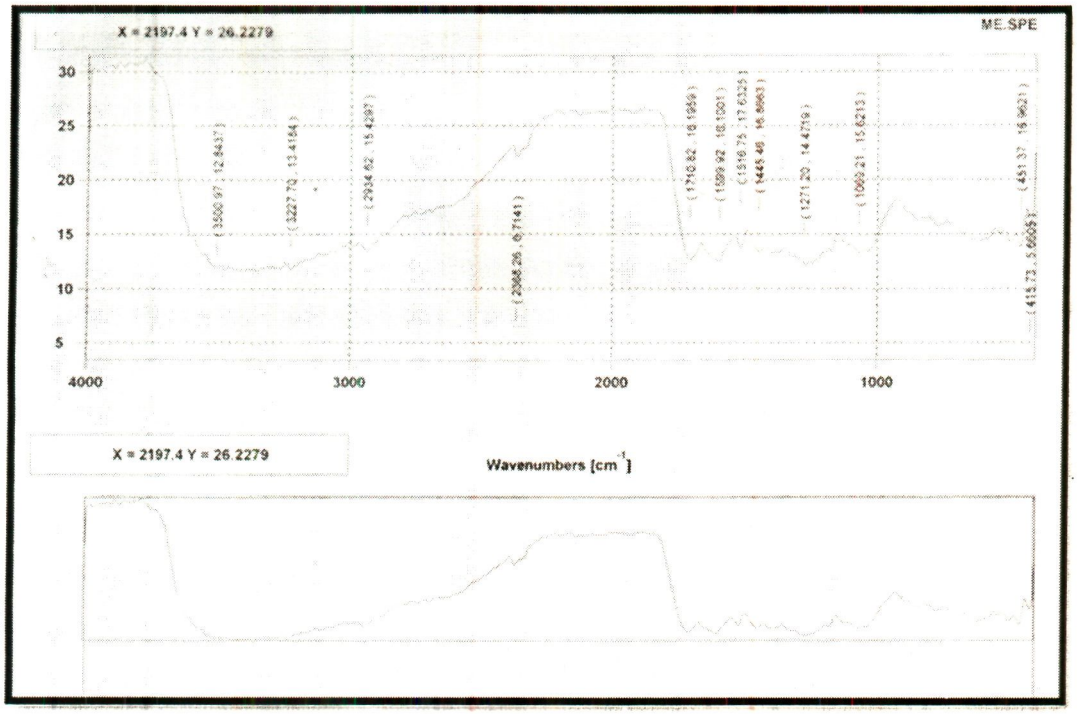
Name of the Fraction	Total phenolic content (mg/g)		
	Mangifera indica	*Psoralea corylifolia*	*Trigonella foenumgraecum*
Petroleum ether	32.2±0.69	43.1±1.03	56.0±1.11
Benzene	44.9±1.14	43.3±1.18	77.7±0.65
Ethyl acetate	454.5±6.37	292.0±2.38	65.4±1.96
Acetone	538.0±3.4	337.3±1.41	132.3±1.58
Methanol	497.6±3.72	367.6±1.53	199.8±2.35

Data are the mean value of three experiments



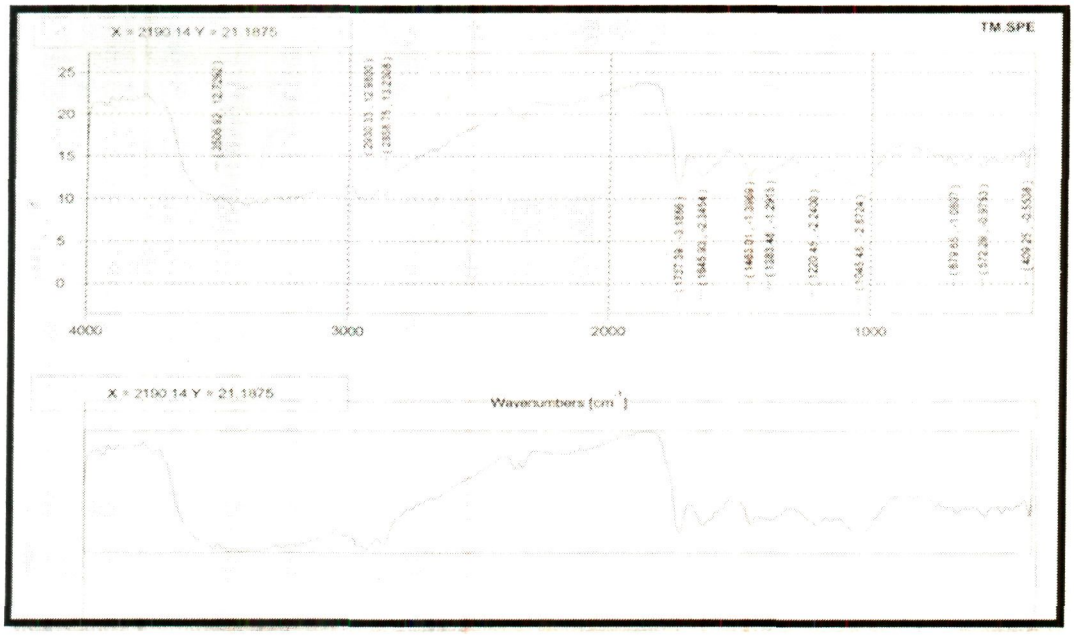
Plant extract (fraction)	Major IR peak position	Inference drawn	Possible compounds presents
Mangifera indica (methanol)	υ (cm^{-1})		
υ(O-H)	3479	Hydroxyl group	Sugar moiety
υ(C-H)	2919	Aliphatic hydrocarbon	Chromones
υ(C=O)	1710	Keto C=O stretching	
υ(C=C)	1513	Aromatic ring stretching	
υ(C-O-C)	1429	Chromone C-O-C stretching	

Figure 51: IR spectroscopic analysis of methanol fraction of *M. indica* (leaf) extract.



Plant extract (fraction)	Major IR peak position	Inference drawn	Possible compounds presents
Psoralea corylifolia (methanol)	υ (cm^{-1})		
υ(O-H)	3500	Hydroxyl group	Flavanoids
υ(C-H)	2934	Aliphatic Hydrocarbon	Coumarin
υ(C=O)	1710	Coumarin carbonyl	
υ(C=O)	1645	Favanoid carbonyl	
υ(C-O-C)	1271	Coumarin C-O-C group	
υ(C=N)	1445	Aromatic ring stretching	
υ(C=C)	1516	Aromatic ring stretching	

Figure 52: IR spectroscopic analysis of methanol fraction of *P. corylifolia* (seed) extract.



Plant extract (fraction)	Major IR peak position	Inference drawn	Possible compounds presents
Trigonella foenum-graceum (methanol)			
υ(O-H)	3506	Hydroxyl group	
υ(C-H)	2930, 2858	Aliphatic hydrogen stretching	Coumarin
υ(C=O)	1737	Coumarin carbonyl group	Nicotinic acid
υ(C=O)	1645	Chromone carbonyl group	sistosterol
υ(C-O)	1220	Coumarin, flavanoid	
υ(C-O-C)	1383	Chromone C-O-C stretching	
υ(C=N)	1463	Pyridine aromatic stretching	
υ(O-H)	1045	Starch OH cellulose	

Figure 53: IR spectroscopic analysis of methanol fraction of *T. foenum-graceum* (seed) extract.

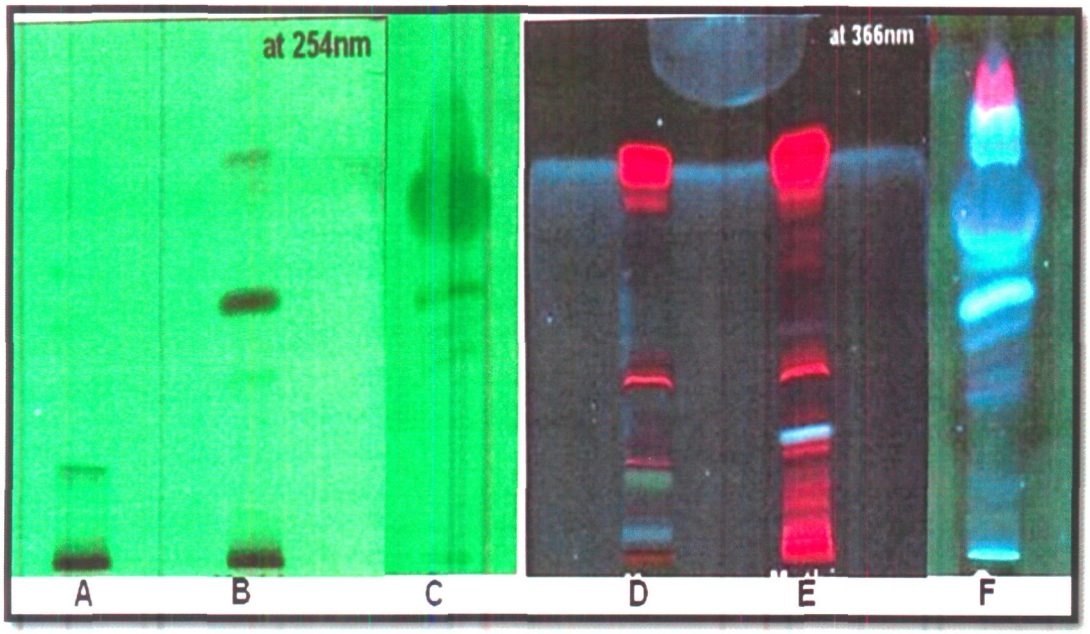


Figure 54: HPTLC chromatogram of methanolic plant extracts. (A,D). *Mangifera indica* (leaf); (B,E). *Trigonella foenum-graceum* (seed) and (C,F). *Psoralea corylifolia* (seed) using chloroform: methanol solvent system.

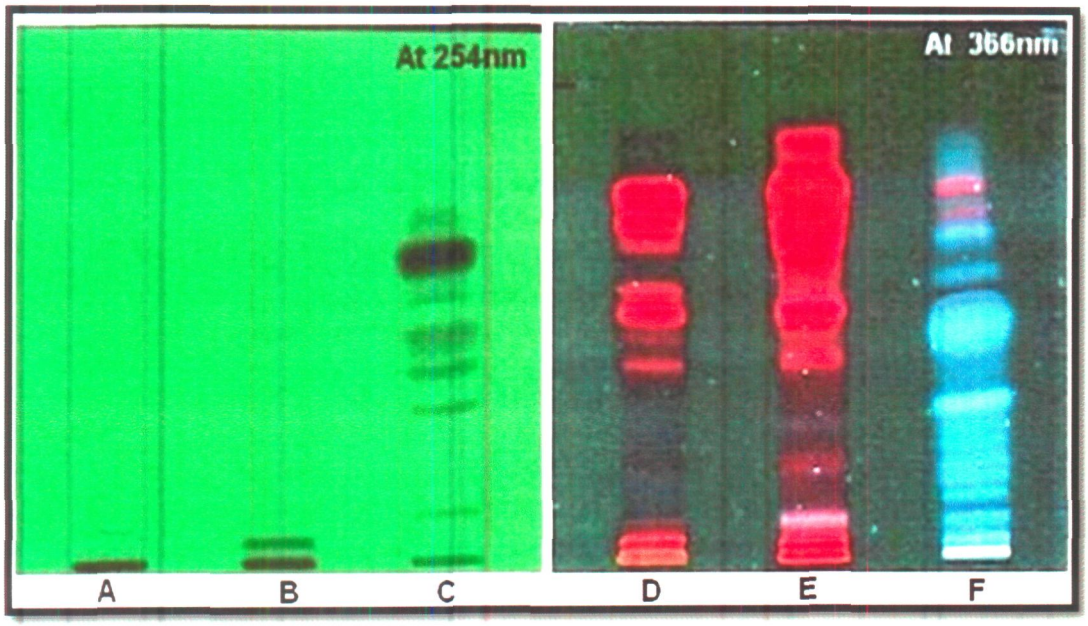


Figure 55: HPTLC chromatogram of methanolic plant extracts. (A,D). *Mangifera indica* (leaf); (B,E). *Trigonella foenum-graceum* (seed) and (C,F). *Psoralea corylifolia* (seed) using toulene: ethyl acetate solvent system.

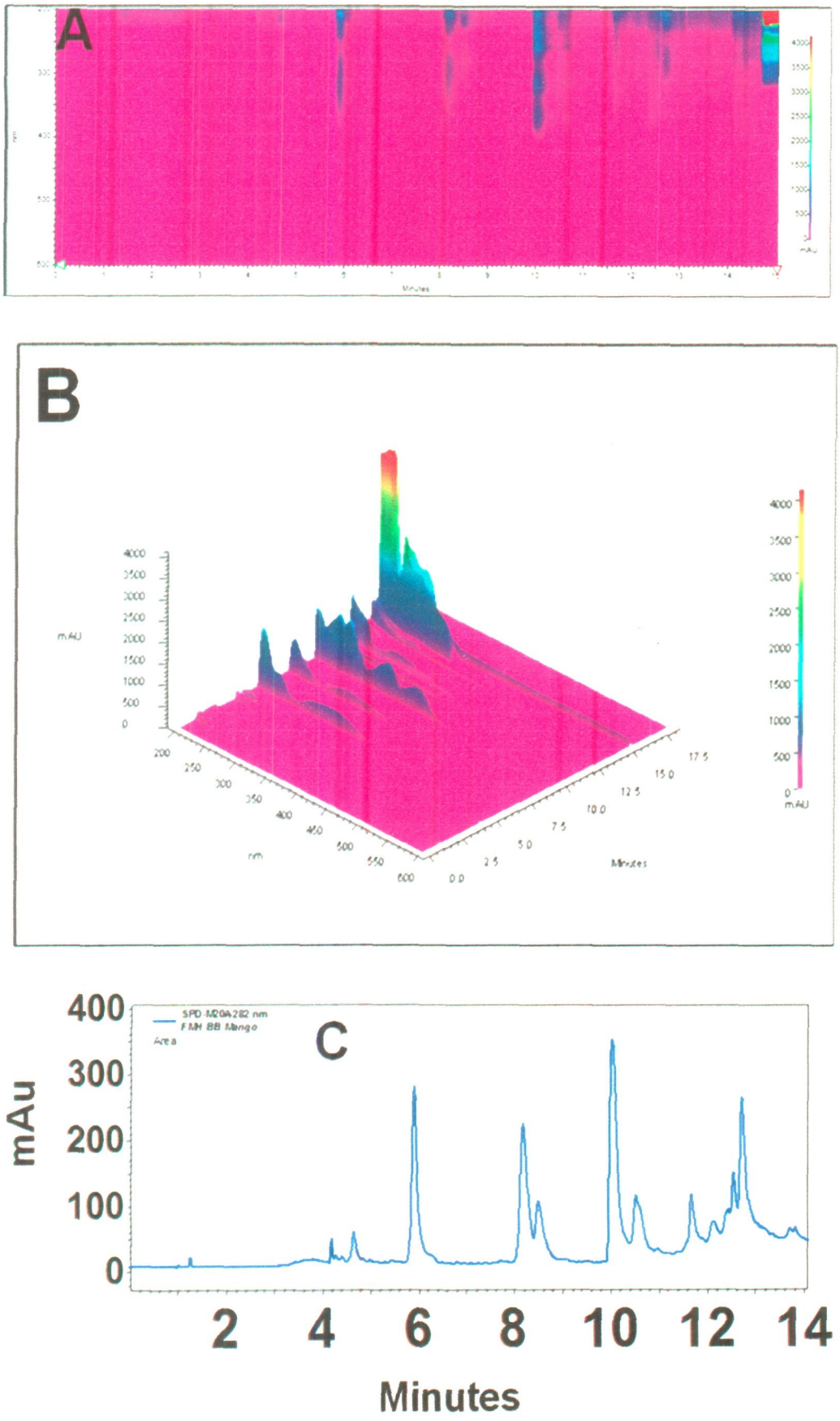


Figure 56: UPLC chromatogram of methanol fraction of *M. indica* (leaf) extract.

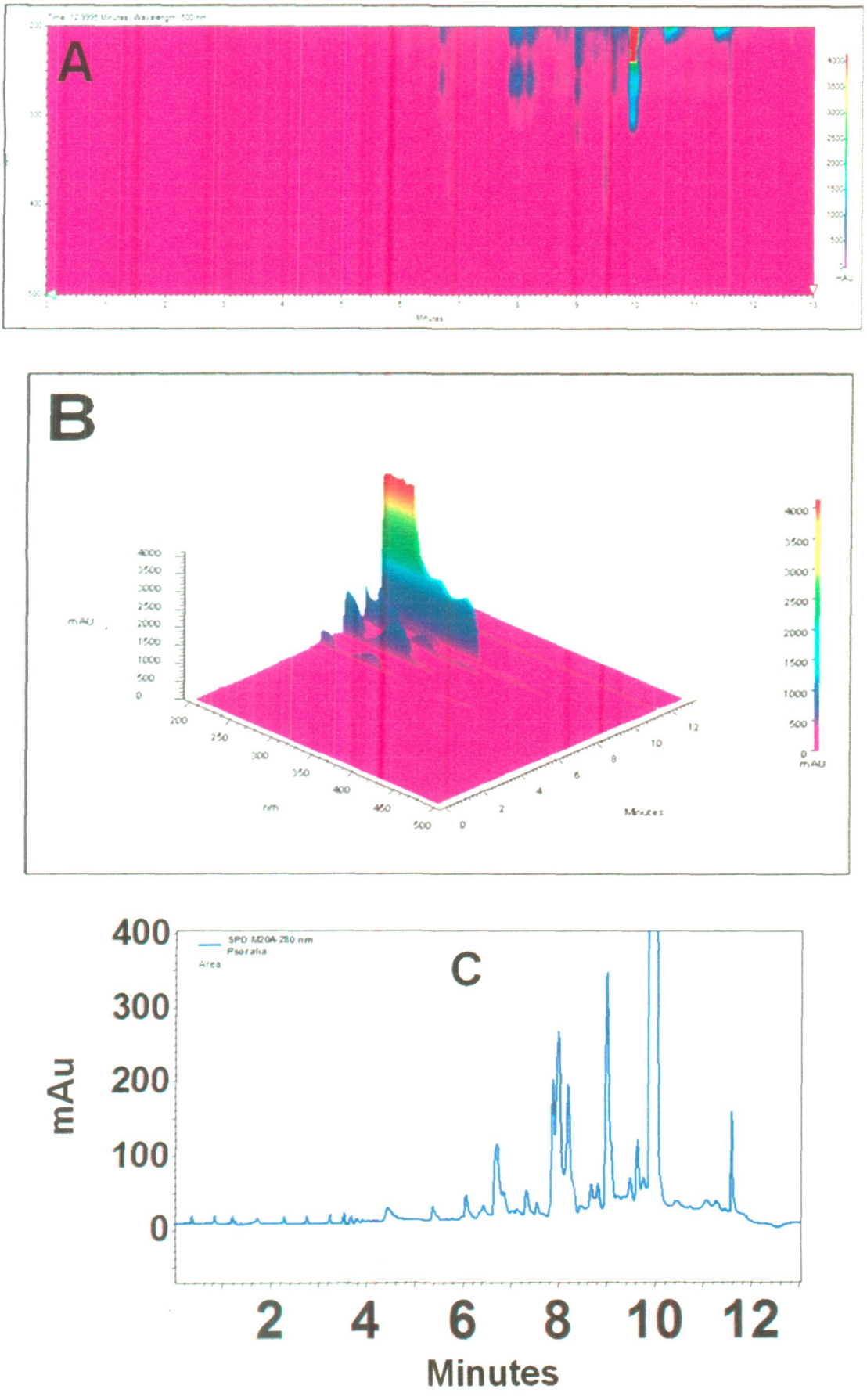


Figure 57: UPLC chromatogram of methanol fraction of *P. corylifolia* (seed) extract.

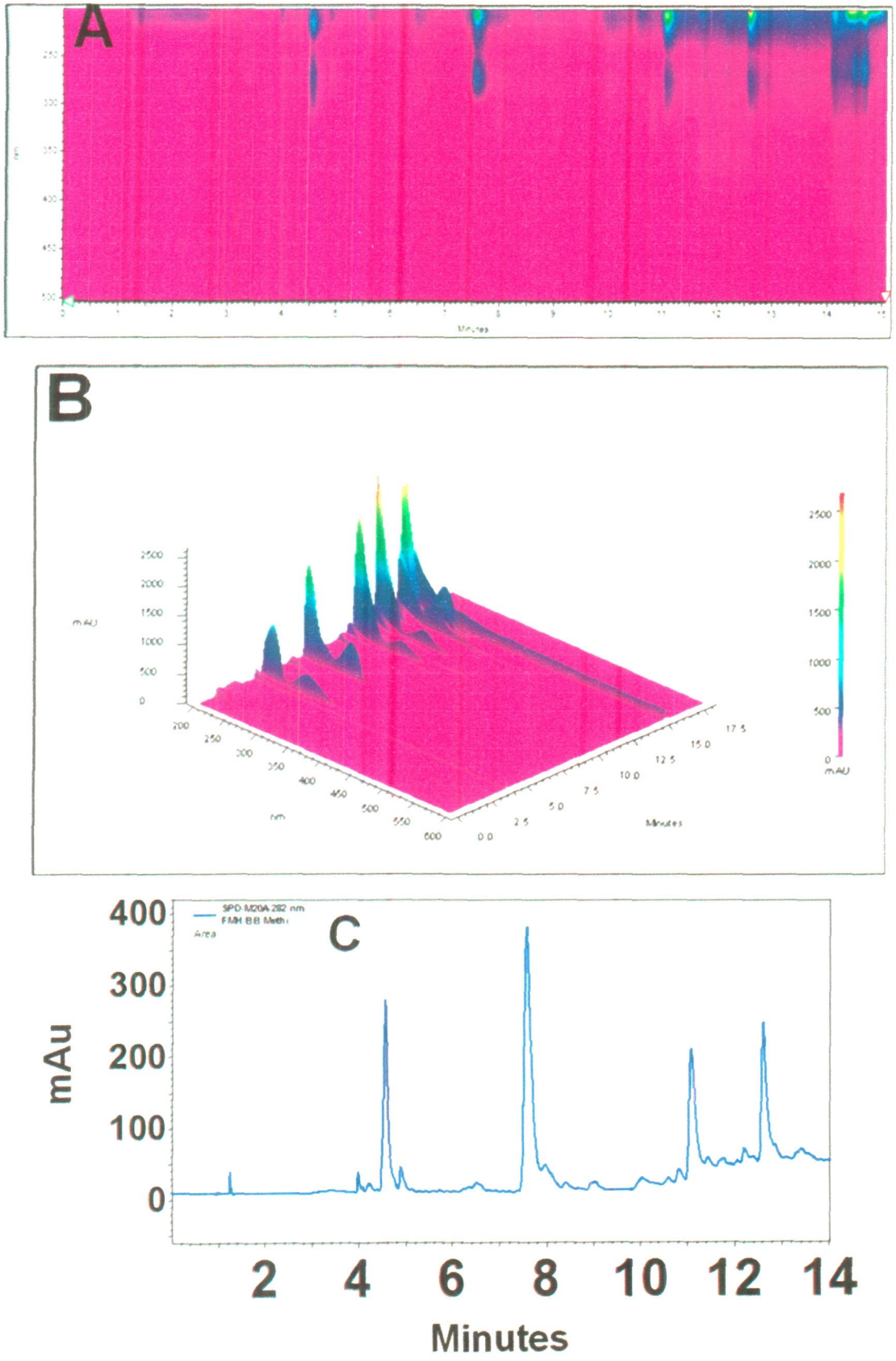


Figure 58: UPLC chromatogram of methanol fraction of *T. foenum-graceum* (seed) extract.

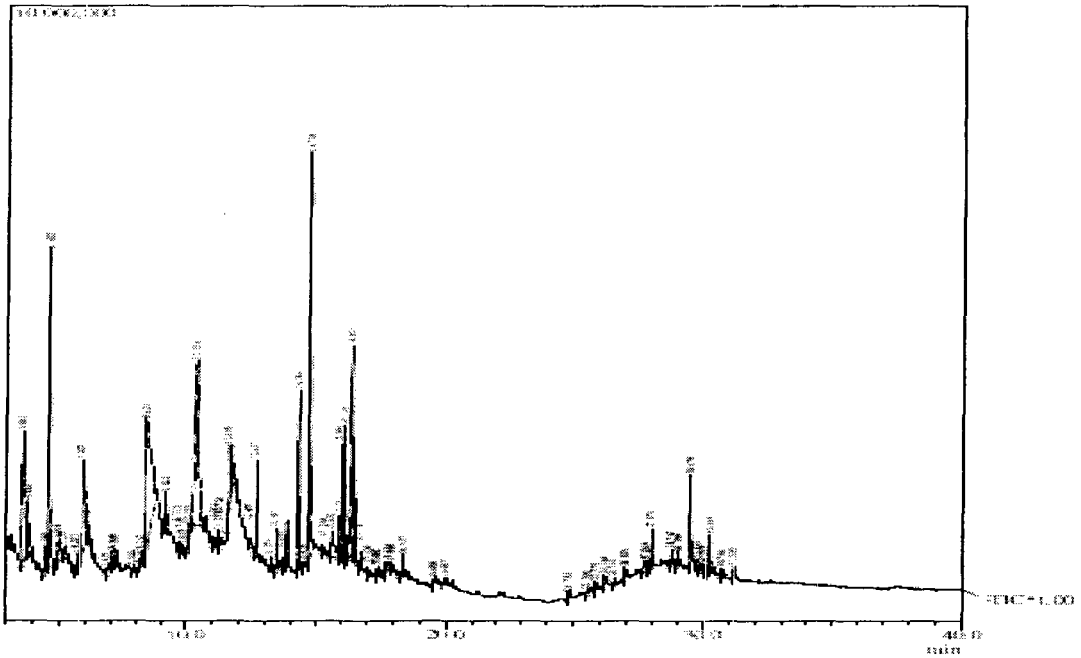


Figure 59: GC-MS chromatogram of methanol extract of *Mangifera indica* (leaf)

Table 44: Components of *Mangifera indica* (leaf) extract as identified by GC-MS analysis.

Peak no.	Components	Retention time	Area (%)
1.	1,3,5-Triazine-2,4,6-triamine	3.66	6.66
2.	1,2,3-Propanetriol, monoacetate	3.88	1.40
3.	4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl	4.60	8.48
4.	2-Furancarboxaldehyde, 5-(hydroxymethyl)	5.90	4.72
5.	1,2,3-Benzenetriol	8.42	15.60
6.	Benzoic acid, 4-hydroxy	10.36	12.09
7.	1,2,3,4,5,6,7,8-Octahydro-2-naphthol, 4-methylene-2,5,5-trimethyl	11.61	1.03
8.	Tetradecanoic acid	12.63	1.72
9.	Pluchidiol	13.17	1.19
10.	Hexadecanoic acid, methyl ester	14.28	2.58
11.	n-Hexadecanoic acid	14.70	9.96
12.	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	15.99	1.71
13.	Phytol	16.11	2.24
14.	9,12,15-Octadecatrienoic acid, (Z,Z,Z)-	16.40	6.22
15.	Stigmast-5-en-3-ol, (3.beta.)-	29.47	2.32
16.	Lupeol	30.26	1.07

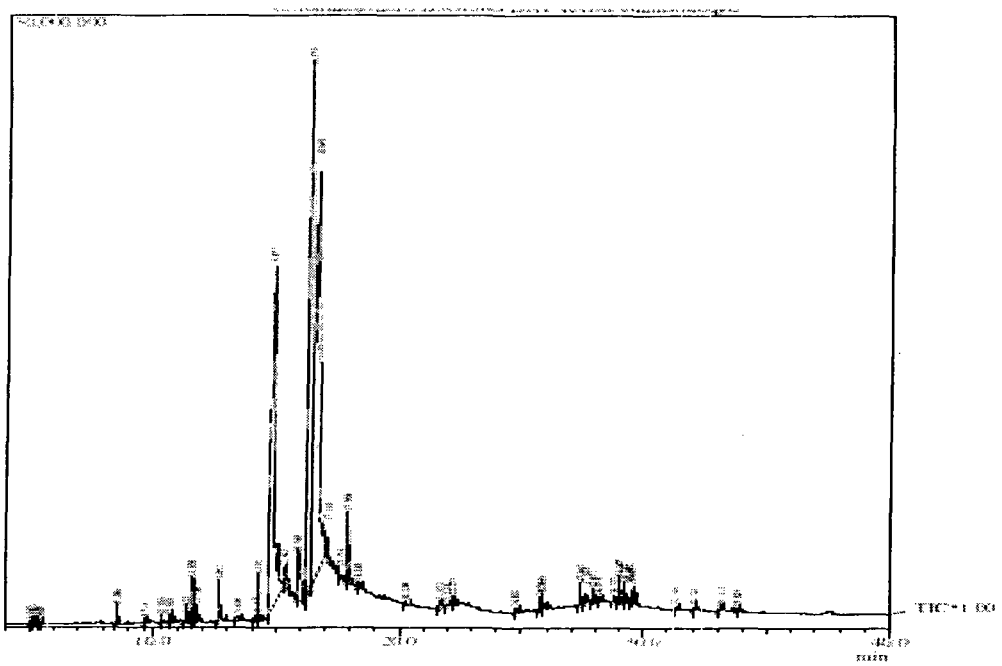


Figure 60: GC-MS chromatogram of methanol extract *Psoralea corylifolia* (seed)

Table 45: Components of *Psoralea corylifolia* (seed) extract as identified by GC-MS analysis.

Peak no.	Components	Retention time	Area (%)
1.	Trans(β)-caryophyllene	8.58	0.22
2.	1-Heptatriacotanol	10.69	0.29
3.	Caryophyllene oxide	11.32	0.48
4.	3-Methyl-5-(2,6,6-trimethyl-1-cyclohexen-1-yl)-1-pentyn-3-ol	11.60	0.64
5.	3-Ethyl-3-hydroxyandrostan-17-one	11.75	0.25
6.	Myristic acid	12.64	1.05
7.	3,7,11,15-Tetramethyl-2-hexadecen-1-ol	13.40	0.35
8.	Palmitic acid, methyl ester	14.29	0.57
9.	Palmitic acid	14.87	23.12
10.	4-[3,7-Dimethyl-3-vinyl-1,6-octadienyl]phenol	16.37	27.73
11.	9,12-Octadecadienoic acid	16.66	35.72
12.	Linalol oxide, trimethylsilyl ether	21.67	0.20
13.	Squalene	24.80	0.11
14.	Hexacosane	25.71	0.20
15.	(+)-cis-Longipinane	25.84	0.42
16.	γ-Tocopherol	27.34	0.34
17.	Thunbergol	27.62	0.37
18.	Cholesteryl myristate	27.87	0.24
19.	Stigmasterol	29.00	0.57
20.	γ -Sitosterol	29.49	0.19
21.	trans-Longipinocarveol	29.60	0.22

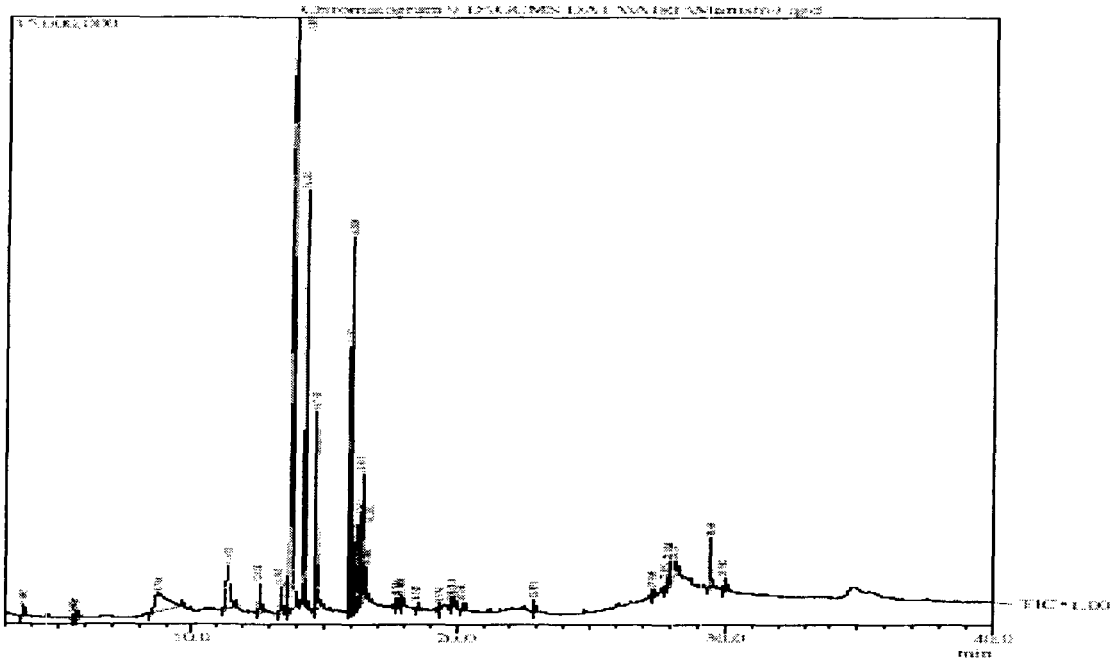


Figure 61: GC-MS chromatogram of methanol extract *Trigonella foenum-graceum* (seed)

Table 46: Components of *Trigonella foenum-graceum* (seed) extract as identified by GC-MS analysis.

Peak no.	Components	Retention time	Area (%)
1.	1,2,3-Benzenetriol	8.76	6.13
2.	Capric acid	11.42	4.20
3.	1,3,7-Trimethyl-3,7-dihydro-1h-purine-2,6-dione	13.85	40.82
4.	Methyl 14-methylpentadecanoate	14.29	8.22
5.	Palmitic acid	14.70	6.41
6.	Linoleic acid, methyl ester	15.93	5.58
7.	9,12,15-Octadecatrienoic acid, methyl ester, (Z,Z,Z)-	16.00	9.17
8.	Phytol	16.11	1.45
9.	Methyl stearate	16.20	1.75
10.	10,12-Hexadecadien-1-ol	16.34	2.56
11.	cis-11-Eicosenoic acid, methyl ester	17.75	0.43
12.	Palmidrol	17.89	0.10
13.	Methyl 18-methylnonadecanoate	17.95	0.12
14.	Hexacosane	18.54	0.11
15.	1-Monopalmitin trimethylsilyl ether	19.37	0.13
16.	Methyl 18-methylnonadecanoate	22.87	0.40
17.	2-Hydroxy-3-(palmitoyloxy)propyl (9E)-9-octadecenoate	28.14	1.48
18.	Stigmasterol	29.46	2.01
19.	Stigmast-8(14)-en-3.beta.-ol	29.98	0.48

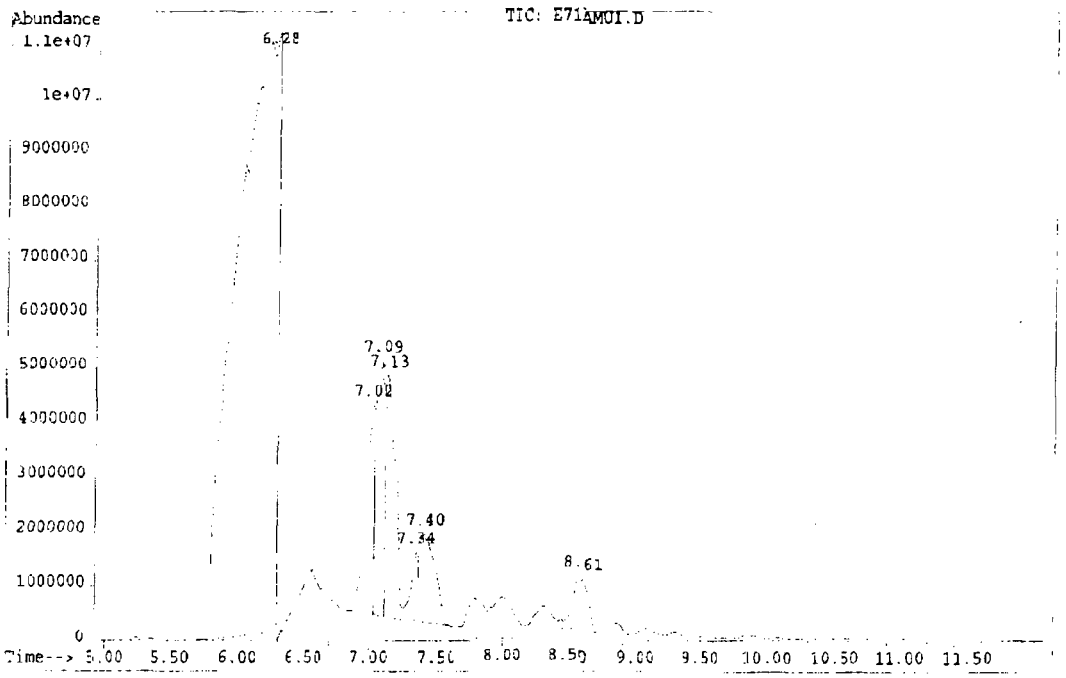


Figure 62: GC-MS chromatogram of *Syzygium aromaticum* (clove) oil

Table 47: Components of clove (*Syzygium aromaticum*) oils as identified by GC-MS

Peak No	Components	Retention time	Area%
1	Eugenol	6.28	74.32
2	β-Caryophyllene	7.03	4.92
3	Iso-caryophyllene	7.09	5.96
4	Napthalene, 1,2,3,5,6,8a-hexahydro-4,7-dimethyl-1-(1-methyl ethyl)	7.14	7.04
5	1,6-Octadiene-ol-,3,7-dimethyl acetate	7.34	1.28
6	α-Caryophyllene	7.40	4.05
7	Caryophyllene oxide	8.61	2.41

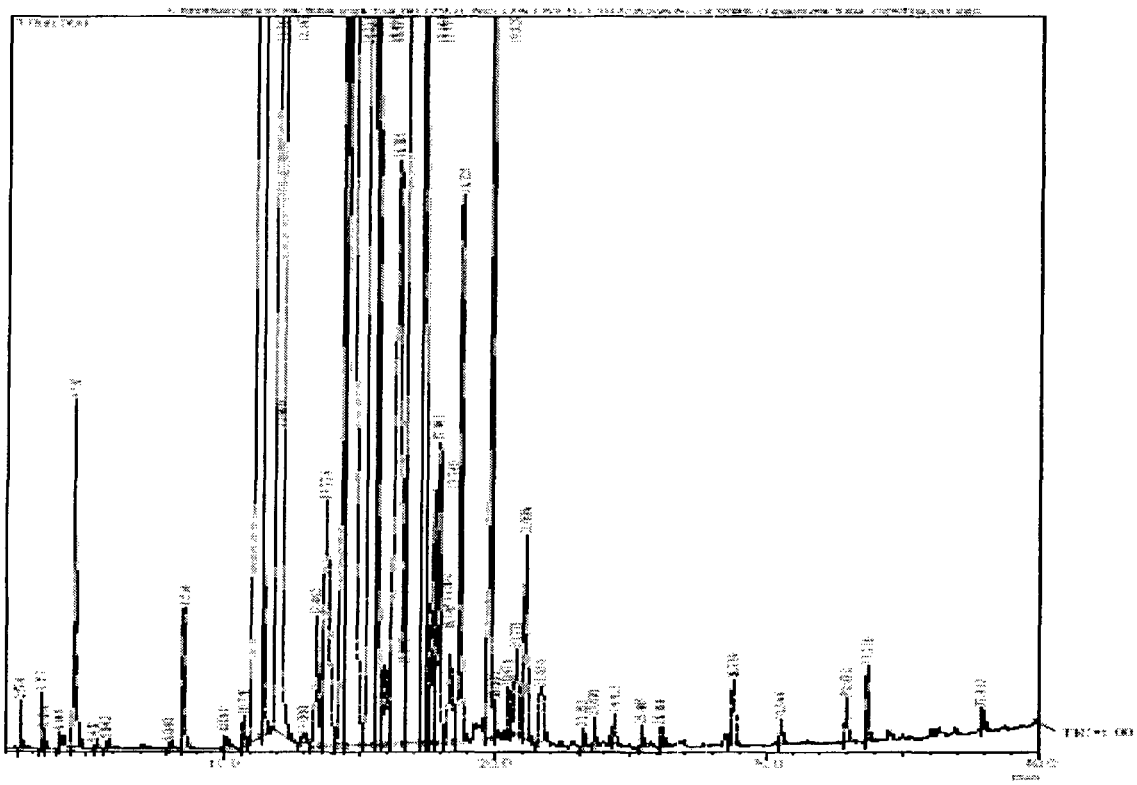


Figure 63: GC-MS chromatogram of *Mentha piperita* (peppermint) oil

Table 48: Components of peppermint (*Mentha piperita*) essential oil as identified by GC-MS analysis.

Peak no.	Components	Retention time	Area (%)
1.	Limonene	4.58	0.53
2.	Menthone	11.36	16.44
3.	Isomenthone	12.14	10.47
4.	1-Hydroxyoctane	13.77	0.84
5.	Isopulegol	14.37	2.16
6.	Menthyl acetate	14.70	7.47
7.	Neoisomenthol	15.43	11.33
8.	Neoisopulegol	15.57	1.84
9.	2-Isopropyl-5-methylcyclohexanol	16.38	2.74
10.	Menthol	17.16	36.87
11.	Pulegone	17.37	1.60
12.	Lavandulol	17.94	0.56
13.	α-Terpineol	18.72	0.99
14.	Piperitone	19.82	2.17

Discussion

6.1. *Pseudomonas* isolates and their characteristics

Control of bacterial infections by inhibiting microbial growth has been the primary approach of antimicrobial chemotherapy. Several classes of antibiotics natural, semisynthetic and synthetic antibacterial drugs are used singly or in combinations. Excessive and indiscriminate use of antibiotics has resulted in selection of resistant bacteria with higher level of tolerance against broad spectrum antibiotics (Hancock, 1998; Lewis, 2001; Harbarth and Samore, 2005). Development of multidrug resistance and its dissemination through various gene exchange mechanisms (horizontal gene transfer) has created immense clinical problems in the treatment of infectious diseases (D'Costa et al. 2006; Martinez, 2008). A large number of such problematic multidrug resistant bacteria (MDR) have been identified by WHO which needs immediate attention (Livermore, 2002; Ahmad et al. 2009; O'Connell et al. 2013).

Pseudomonas aeruginosa is an opportunistic pathogen responsible for 57% of total nosocomial infections such as infections of respiratory system and urinary tract infections (Oncul et al. 2009). *P. aeruginosa* is the major cause of secondary infections in immunocompromised patients with cystic fibrosis, burn wound and HIV causing maximum morbidity and mortality (Vandeputte et al. 2010). *P. aeruginosa* has number of resistance mechanism and forms biofilm which further enhances its tolerance level several hundred folds and therefore the pathogen becomes difficult to eradicate with currently used antibiotic therapy in many cases of infection (Klausen et al. 2003). To facilitate the establishment of infection, *P. aeruginosa* produces both cell-associated and extracellular virulence factors globally regulated by well defined quorum sensing systems arranged in hierarchical manner with las system at the top, positively controlling the activity of rhl system (Jimenez et al. 2012). The las system utilizes N-(3-oxododecanoyl)-L-homoserine lactone (3-oxo-C12-HSL) whereas rhl system functions by means of N-butanoyl-Lhomoserine lactone (C4-HSL) as the signal molecules (Rasmussen and Givskov, 2006). Intermediate between the two is the quinolone system which utilizes 2-heptyl-3-hydroxy-4-quinolone as the signal molecule (Dekimpe and Deziel, 2009). Pyocyanin and rhamnolipids production is controlled by rhlIR system whereas elastase and proteolytic activities by lasIR system (Senturk et al. 2012).

In the present study we have isolated *Pseudomonas aeruginosa* from clinical and environmental sources and some other Gram negative bacteria (*Aeromonas* and *Klebsiella*) to assess their drug resistance pattern and virulence factors including biofilm formation and AHL detection. The aim was to obtain most relevant strain to be used in our screening studies along with standard strains for quorum sensing (QS) interference by natural products.

A total of 47 *Pseudomonas*, 6 *Klebsiella* and 2 *Aeromonas* isolates were subjected to drug sensitivity which revealed that majority of the isolates were resistant to many drugs indicating their multiple drug resistance nature. These drug resistant isolates might have various mechanisms of resistance known for gram negative bacteria including *Pseudomonas aeruginosa* (Alanis, 2005; Nikaido, 2009).

These isolates were further subjected for detection of AHLs using *Chromobacterium violaceum* CVO26 and or *Agrobacterium tumefaciens* A136 biosensor strains. A total of 29 strains produced AHL as evidenced from the pigment production by CVO26. This strain could detect exogenous short chain AHLs and produce violacein pigment. Further the strains also produced 3-oxo-C12-HSL as detected by the *Agrobacterium tumefaciens* A136. This strain is very sensitive and could detect the presence of long chain AHLs ranging from C6 to C14 types (Mclean et al. 2004). TLC analysis revealed that a total of seven *Pseudomonas* isolates showed the presence of C6-HSL while C4-HSL was also detected in all the isolates of *Pseudomonas*. Production of 3-oxo-C12-HSL was detected in 9 of the *Pseudomonas* isolates. Production of C4 and C6-HSL is expected while certain strains could not produce 3-oxo-C12-HSL. Variations in the production of various types of AHLs are also reported by other workers demonstrating similar results (Ravn et al. 2001; Kumar et al. 2011; Gowda et al. 2013). *P. aeruginosa* has two LuxIR homologue pairs, LasIR and RhlIR, which produce and detect the AHL signaling molecules 3-Oxo-C12-HSL and C4-HSL, respectively. The LasR-3-Oxo-C12-HSL complex induces expression of LasI as well as expression of RhlR, thereby positively regulating both AHL-based QS systems. The RhlR-C4HSL complex subsequently mediates its own autoinduction but has no direct effect on the LasR system. LasR and RhlR additionally positively and negatively regulate, respectively, expression of genes involved in the third quorum-sensing loop, which is based on the autoinducer

PQS. PQS autoinduces its own production while concomitantly enhancing RhlR expression, thereby self-limiting its expression via an extended negative-feedback loop. PQS has no direct regulatory activity on the Las QS system (LaSarre and Federle, 2013). However, in the strains under study other AHLs and signal molecules might also have been detected if other specific biosensor strains and or purified standard AHL would have been available. *Aeromonas* strains isolated in the present study also demonstrated the production of C4 and C6-HSL. TLC analysis demonstrated the presence of 3-oxo-C12-HSL in *Aeromonas* WAF47 which was further verified by the results of MS analysis. Earlier study indicated that *Aeromonas hydrophila* produces two types of AHLs, namely N-3-butanoyl-DL-homoserine lactone (C4-HSL) and N-3-hexanoyl-DL-homoserine lactone (C6-HSL), of which C4-HSL was the predominant type (Swift et al. 1997, 1999; Khajanchi et al. 2009; Haslan and Kimiran-Erdem, 2013). Production of AHLs other than C4 and C6-HSL in the cilinical strains of *Aeromonas* has been demonstrated by Chan et al. (2011) which is in agreement with our findings.

P. aeruginosa QS system, consisted of inducer and regulator proteins of las and rhl components which works interdependently in a hierarchal manner to regulate the expression of various genes including virulence ones (Wagner et al. 2007; Siehnela et al. 2010). In the present study, effect of individual QS inducer or regulator component on phenotypic expression of various virulence factors was observed. It is interesting to note that most of the isolates produced virulence factors that are known to be regulated by QS indicating active QS system in these strains. Most bacterial communities grow in 3-dimensional biofilm structures on surfaces in natural, clinical, and industrial settings (Hall-Stoodley et al. 2004). Biofilms consist of a single or multiple species of bacteria that are imbedded in an extracellular polymeric substance (EPS) composing of polysaccharides, proteins, and nucleic acids (Mayer et al. 1999). EPS attaches biofilm cells firmly to surfaces and protects them from harsh conditions. One noticeable feature of biofilm cells is increased resistance to antibiotics (Flemming et al. 2007). Possible reasons of this feature that may be due to the EPS layer include the limitation of the transport of the agents to interior bacterial cells in thick layers and the reduction of available agents by adsorption into or reaction with the EPS matrix (Davies, 2003). The ineffectiveness of antibiotic treatment in the

biofilm diseases may cause serious problems in the eradication of infections (Kociolek, 2009).

Further biofilm screening results showed varying levels of biofilm formation by the isolates in microtitre plate assay. These findings indicated that irrespective of the nature/source of isolation *P. aeruginosa* are resistant to many antibiotics and produce QS regulated virulence factors and form biofilm of varying degree. Similar results were demonstrated by Gupta et al. (2011) who investigated the contribution of QS to the phenotypic expression of multiple virulence factors in clinical isolates of *P. aeruginosa in vitro*. Our findings on isolated strains clearly indicated that QS regulated expression of elastase, total protease, EPS production, swarming motility and established the role of QS in biofilm formation. The characteristics of *Pseudomonas aeruginosa* for AHL production, virulence factors and biofilm formation are in agreement of reports of various workers (Williams, 2007; Antunes et al. 2010; Gupta et al. 2011; Rutherford and Bassler, 2012; Gowda et al. 2013). Although variations in results of resistance profile, AHL detection and other traits is expected due to different nature of *P. aeruginosa* isolates, antibiotics use, environmental conditions and assay systems used for detection and characterization of various traits (Kalia, 2013).

Two strains were selected from above isolates for anti-QS screening of natural products were identified by 16s rRNA gene sequence analysis and identified as *Pseudomonas aeruginosa* PAF79 and *Aeromonas hydrophila* WAF38. These two strains have been included along with standard strains in QS interference studied.

6.2. Screening and evaluation of natural products (antibiotics, plant extracts and essential oils) for their QS interference properties

The lung of cystic fibrosis (CF) patients has a unique susceptibility to chronic *Pseudomonas aeruginosa* infection, which is still the major cause of morbidity and mortality in these patients (Davies, 2002). Although in past decades antibiotic therapy has greatly increased life expectancy, only limited therapeutic options are available, and chronic *P. aeruginosa* infection is rarely eradicated (Gibson et al. 2003). A serious side effect of antibiotic therapy is the development of resistance to the antibiotics used (Livermore, 2002; Andrade et al. 2003; Flamm et al. 2004). *P. aeruginosa* forms biofilms during the infection process, which adds to the difficulties

of eradicating infections by antibiotic intervention, since bacterial cells living as biofilms are much more tolerant to antibiotics than their planktonic counterparts (Costerton et al. 1999; Skindersoe et al. 2008).The increase in antibiotic resistance seen in gram negative bacteria including *Pseudomonas aeruginosa* in clinical and hospital environmental isolates (Ciofu et al. 1994) and their ability to form persistent infection through biofilm formation (Costerton et al. 1995; Costerton, 2001) have drawn attention to the improvement of current treatment strategies. Great efforts have been made to develop antipathogenic drug strategies especially by menas of reducing bacterial virulence through interference of intercellular communication (QS). The mechanisms of action in chronic *P. aeruginosa* infection remain obscure (Wozniak and Keyser, 2003). Immunomodulatory effects and quorum sensing based *P. aeruginosa* virulence factor interference are possible mechanisms (Tateda et al. 2001; Nalca et al. 2006). Blocking of QS in *P. aeruginosa* by the use of QSIs has been shown to be the promising strategy for the treatment of infections (Hentzer et al. 2003; Rasch et al. 2004; Kalia, 2013).

6.2.1. Screening and evaluation of antibiotics as anti-QS agents

Quorum sensing (QS) and is a very sophisticated mechanism by which signal molecules act as autoinducers and trigger a variety of biological functions when microbial populations attain certain cell densities. The QS system in *P. aeruginosa* comprises two hierarchically organized systems, each consisting of an autoinducer synthetase (LasI/RhlI) and a corresponding regulator protein (LasR/RhlR). QS controls not only virulence factor production but also biofilm formation in *P. aeruginosa* (Davies et al. 1998) and thus contributes significantly to pathogenesis and persistence of infection. Since QS plays a key role in the expression of virulence and the interaction with host protection, inhibitors of QS have been suggested to be important components of future antipseudomonal therapies (Ratjen, 2001).

In the present study we have made an attempt to screen a variety of antibiotics at recommended potency for sensitivity determination to assess possible quorum sensing interference using indicator strains of *Chromobacterium violaceum* (CV12472 and CVO26) which produces violacein (a violet pigment) under the effect of quorum sensing. Our findings demonstrated that in addition to known control agent Azithromycin, some other class of antibiotics (Ciprofloxacin, Ceftazidime,

Doxycycline, Erythromycin, Kanamycin and Tobramycin) could significantly inhibit violacein production. Based on the screening studies Doxycycline and Ceftazidime were tested for their interference in QS regulated traits of *Pseudomonas aeruginosa* PAO1 and two other laboratory strains (*Pseudomonas aeruginosa* PAF79 and *Aeromonas hydrophila* WAF38). Doxycycline and ceftazidime exhibit diverse mechanisms of antimicrobial action and are structurally unrelated. Doxycycline targets protein synthesis while ceftazidime inhibits cell wall synthesis via affinity for penicillin-binding proteins (PBPs). Thus it is apparent that the two antibiotics probably do not inhibt QS regulated functions by above mechanisms. We have carefully selected the sub-MICs of these antibiotics which do not inhibit bacterial growth.

The extent of inhibition of violacein was determined by the extraction of violacein pigment from CVO26 in the presence and absence of doxycycline. Doxycycline at sub-MICs (1-4 μg/ml) exhibited concentration-dependent pigment inhibitory activity and which ranged from 40.4-70% ($p \leq 0.005$). Inhibition of QS controlled functions like elastase, total protease, pyocyanin production, chtinase activity, EPS production and swarming motility was ≥60% over control at 4 μg/ml concentration. The observation clearly indicated QS interference by the antibiotics. However, certain virulence factors were significantly inhibited even at lower concentration ranging from (0.5-2 μg/ml). Likewise, biofilm formation was also inhibited considerably at all tested sub-MICs (40.3-79.1%). Doxycycline at selected sub-MICs (4-32 μg/ml) almost resulted in similar inhibition of virulence factors as well as biofilm formation in PAF79 isolate. The significant inhibition of all tested virulence factors at the highest sub-MIC concentration of 32 μg/ml indicated that the effect of the doxycycline is exhibited at higher sub-MICs against doxycycline resistant isolates. Inhibition of QS regulated virulence factors and biofilm by doxycycline is not reported previously so this is probably the first report on QS inhibitory activity of doxycycline.

Further, doxycycline inhibited biofilm formation (71.5%), total protease (66.9%) and EPS production (51%) in *Aeromonas hydrophila* WAF38 at 8 μg/ml of concentration. Doxycycline interference in QS regulated traits in *Pseudomonas aeruginosa* PAO1, PAF79 and *Aeromonas hydrophila* WAF38 indicates a broad-

spectrum activity although the mechanism of action is difficult to predict. However, effect of sub-MICs of doxycycline on beta-galactosidase activity of *E. coli* MG4/pKDT17 containing *lasB'-lacZ* detection system by which the lasB promoter driven lacZ expression by LasR is monitored and the *lasR* gene is under the control of the *lac* promoter indicated direct interference with QS system. The findings of the assay clearly point out the decrease in the levels of AHL upon treatment with doxycyline. Therefore, reduced levels of AHL caused decreased expression of *lasb* gene as evident by the decreased β-galactosidase activity.

Early workers have demonstrated the effect of sub-MICs of tetracycline on the surface expression of *Pseudomonas aeruginosa* ferripyochelin binding protein (FBP) (LeVatte et al. 1990). The authors provided evidence that sub-MIC of antibiotic inhibits FBP surface expression as well as proteolytic activity *in vitro*. However, tetracycline had no effect on the activities of exotoxin A or exoenzymes S. Although at the time of the above report the role of QS was not clearly known.

Other antibiotic tested for QS interference is ceftazidime a β-lactam antibiotic. Since violacein production in CVO26 is induced in the presence of AHL, inhibition of violacein production at sub-MICs of ceftazidime indicated the interference in AHL based QS in *Chromobacterium violaceum*. Efficacy of ceftazidime at sub-MICs (0.06-0.5 µg/ml) was assessed in reducing the QS regulated virulence traits in PAO1. Virulence factors were inhibited significantly (55.7-81.7%) over control at the highest tested sub-MIC (0.5 µg/ml). Interestingly, maximum reduction was recorded for motility followed by biofilm formation, chitinase activity, elastase activity, production of Pyocyanin and EPS and total protease activity. Inhibition of several QS regulated traits simultaneously is a clear confirmation of QS interference by ceftazidime at important regulatory point of QS-systen of PAO1. Our findings are in agreement with Skindersoe et al. (2008). They demonstrated QS modulatory role of three antibiotics namely azithromycin, ciprofloxacin and ceftazidime. The effect of ceftazidime on *Pseudomonas aeruginosa* QS regulated virulence factors was analyzed by DNA microarray, the result clearly showed decrease of a range of QS regulatory virulence factors such as elastase, chitinase, protease and pyocyanin production. The possible mechanism suggested is due to the change in membrane permeability which might influence the influx of AHL molecule.

In the last few years other workers have also demonstrated QS modulatory activity of antibiotics such as tobramycin (Babic et al. 2010). They suggested that antibiotics inhibited RhlI/R system by reducing levels of C4-HSL production but this effect was not due to decrease of rhlI transcription and required tobramycin-ribosome interaction. The present study revealed a concentration dependent decrease in the levels of AHL produced by PAO1. The reduced AHL levels leads to decreased transcriptional activation of *lasB* in *E. coli*, which indicates that ceftazidime inhibits the *las* system. Biofilm inhbition by the antibiotic at sub-MIC might be at the developmental stage as well as maturation as the drug is added at the beginning of the assay which might be the result of the disturbance caused by interbacterial binding. These antibiotics due to this additional activity might be having therapeutic advantage over other drugs in treating biofilm based infections (Bala et al. 2011).

Thus, our findings could be compared with above studies in assessing the QS interference properties of doxycycline and ceftazidime against the *P. aeruginosa* (PAO1, PAF79) and *Aeromonas hydrophila* (WAF38).

Antibiotics Doxycycline and Ceftazidime are structurally very different from all non antibiotic QSIs described until now; moreover, like many other antibiotics, they are very bulky molecules. *In silico* docking of azithromycin, ceftazidime, and ciprofloxacin to the ligand binding domain of the LasR protein showed that the antibiotics have a low affinity for the LasR receptor site, mainly due to spatial penalties (Skindersoe et al. 2008). This supports the idea that antibiotics may exert their QS regulatory effects through mechanisms different from those of the other QSIs, thereby opening new and interesting possibilities for combination therapy with QSIs with different modes of action. The usefulness of the combination of QSIs and traditional bactericidal compounds has been proved by several *in vitro* experiments (Davies et al. 1998, Hentzer et al. 2003, Rasmussen et al. 2005b). The understanding of some antibiotics' dual course of action may have the potential to further add to therapies against pathogens in which the key element attenuates rather than kills the bacteria directly. This strategy seeks to allow the host defense system to eliminate the attenuated bacteria (Skindersoe et al. 2008; Bala et al. 2011).

6.2.2. Screening and evaluation of plant extracts for QS interference activity

Medicinal plants living in an environment with a very high bacterial cell density were long suspected to have protective mechanism against microbial infection (Mclean et al. 2004; Vattem et al. 2007). Recent concept and primitive knowledge on plant microbiome, rhizobiome and plant probiotic concept is now slowly gaining momentum in plant research. The plants are well known to produce secondary metabolites and compounds in root exudates etc. with a variety of biological activities due to diverse phytochemical constituents. The plants which are dietry or used traditionally for treatment of various ailments including infectious diseases are supposed to be safe and non-toxic (Vattem et al. 2007). It is therefore expected to get novel and safe bioactivities from natural products of plant origin. We have previously screened a number of Indian medicinal plants for antibacterial (Ahmad et al. 1998; Ahmad and Beg, 2001; Aqil and Ahmad, 2007), antifungal (Aqil and Ahmad, 2003), antioxidant (Aqil et al. 2006) and antimutagenic activities (Zahin et al. 2010a). In this study a total of 37 plant extracts prepared in methanol, extracted, dried and resuspended in DMSO to test their QS interference activity against *Chromobacterium violaceum* 12472 and CVO26 strains at 1000 µg/ml. We have not considered very higher concentrations because current plant extract screening strategy demands activity at lower concentrations i.e. ≤1000 µg/ml to be considered potentially active. Results of the screening with CV 12472 showed that out of the 37 plants tested, 10 plants demonstrated varying levels of violacein interference which included maximum inhibition/interference by methanolic extract of *Mangifera indica* (leaves) followed by *Plumbago zeylanica* (root), *Camelia sinensis* (leaf), *Psoralea corylifolia* (seed), *Trigonella foenum-graceum* (seed), *Terminalia chebula* (fruit), *Cuminum cyminum* (fruit), *Holarrhena antidysenterica* (bark), *Delonix regia* (flower) and *Lawsonia inermis* (leaves). However, when tested in CVO26, three plant extracts gave similar result as in CV12472 while the other extracts showed relatively less violacein inhibition although we had not expected such a variation. Due to more intense and consistent activity these plants were selected for further study.

Violacein production in *C. violaceum* is regulated by CviIR-dependent QS system. Therefore, any inhibition of pigment in CVO26 is a direct evidence of QS interference. Remaining extracts found inactive in this assay system might exhibit

some level of activity in the other assay systems. Since CV based screening is biased towards only AHL, therefore, these plants which are found inactive need to be either tested using other test assay systems to explore potential anti-QS activity or by modifying the extraction in other solvent systems.

Our Screening results are comparable with a number of screening results in other plants reported by various (Adonizio et al. 2006; Vattem et al. 2007; Adonizio et al. 2008a; Huerta et al. 2008). Some of the other plants like *Cuminum cyminum* and *Terminalia chebula* have also been reported for varying levels of QS inhibitory activity by Abraham et al. (2012) and Sarabhai et al. (2013), respectively. However, the variation in the studies with plant extracts is primarily due to the adoption of different methods for extraction, nature of material used for extraction, concentration used and microbial test assays applied by different workers.

6.2.3. Fraction based QS interference study of three selected plants

We have selected three plants based on preliminary screening results to study fraction based activity. Sequential fractionation in petroleum ether, benzene, ethyl acetate, acetone and methanol was made and tested again for QS interference using CV12472 strain. Pigment inhibition was significant only in methanol fraction and little activity was found in acetone and ethyl acetate fractions of *Mangifera indica* (leaf) at higher concentrations tested. Similar trend of QS inhibitory activity was also detected in *Psoralea corylifolia* (seed) extract. Conversely, methanol fraction of *Trigonella foenum-graceum* (seed) could enhance the pigment production indicating interference of QS possibly by up regulating violacein phenotype. This peculiar behaviour was checked several times to ensure the enhancement in the extract and recorded.

6.2.4. Screening and evaluation of essential oil for QS inhibitory activity

We have previously reported antibacterial, antifungal, antioxidant as well as antimutagenic activities of plant essential oils (Ref). In this study we screened 21 common essential oils against *Chromobacterium violaceum* to assess their anti-QS activity by observing violacein inhbition. Interestingly essential oil of clove, peppermint, lavender and cinnamon could inhibit violacein production in both wild type (CV1272) and mutant (CVO26) strain to varying levels. However, other oils tested at their respective sub-MICs could not interfere with violacein production.

However, it is possible that these oils may exhibit QS interference in other bacterial biosensor strains having different QS system and needs to be explored further. In the recent years many workers have reported QS interference in oils of rose, geranium, lavender and rosemary (Szabo et al. 2010), in Colombian essential oil of *Lippia alba*, *Ocotea* sp, *Elettaria cardamomum*, *Swinglea glutinosa*, *Myntotachys mollis* and *Zingiber officinale* (Jaramillo-Colorado et al. 2012), in essential oils of rose, clove, chamomile (Eris and Ulsoy, 2013) using one or other QS reporter strains.

Our findings are in agreement with the reports of above workers. However, variations even in the same oils are sometime expected due to the chemical diversity, quantity, composition vary due to source of oil, extraction procedure, variety of plants used and quality of essential oil available.

6.3. Effect of most active fractions of plant extract on QS-regulated virulence factors of *Pseudomonas aeruginosa* and *Aeromonas hydrophila*

In the present study we have used two strains of *P. aeruginosa* (PAO1, a well studied strain for QS and a lab isolates PAF79). Few QS regulated characteristics were also tested in *Aeromonas hydrophila* WAF38 strain.

In this study methanol fraction of the plant extract was found to be the most active in CVO26 and therefore selected for *in vitro* efficacy against *Pseudomonas aeruginosa* (PAO1 and PAF79) and *Aeromonas hydrophila* WAF38. Methanolic extract of *M. indica* leaf extract demonstrated significant ($p \leq 0.005$) decrease ($\geq 70\%$) in elastase, pyocyanin, swarming motility and above 50% reduction in EPS, total protease and chitinase activity in PAO1 strain. Almost similar effect was also observed in PAF79 where protease was inhibited upto 82%. Interestingly biofilm formation was maximally inhibited in *A. hydrophila* followed *P. aeruginosa* strains.

P. aeruginosa proteases and LasB are believed to play a major role in pathogenesis via host tissue degradation (Kessler et al. 1998). The virulence factor LasB (elastase) is generally thought to be under the control of the *lasI-lasR* system (Hentzer and Givskov, 2003); however, *rhlI-rhlR* also controls activity to a lesser extent (Brint and Ohman, 1995; Pearson et al. 1997). *M. indica* extract resulted in significant decrease ($p \leq 0.005$) in elastase and protease activites of PAO1. This is in agreement with earlier studies of Adonizio et al. (2008a) and Abraham et al. (2012). It

is likely that the extract down regulated LasB activity. According to a study garlic extract (at 2% w/v) show a 50% decrease in LasB activity (Rasmussen et al. 2005a), where as purified halogenated furanone from *D. pulchra* (10 μM) induced almost 90% decrease (Hentzer et al. 2003). In the recent years reports have appeared for many plants showing elastase inhibition activity to varying levels (Vandeputte et al. 2010; Singh et al. 2012). Significant elastase inhibition has also been reported in six south florida (Adonizio et al. 2008a). Plant extracts of *Ananas comosus*, *Musa paradiciaca*, *Manilkara zapota*, *Ocimum santum* and *Lagerstroemia speciosa* demonstrated elastase inhibitory activity of varying degree (Musthafa et al. 2010; Singh et al. 2012). The elastase and total protease activity are similarly inhibited by the methanol fraction of *Psoralea corylifolia* (seed) but the decrease was less in comparison to *M. indica* at their respective sub-MICs. On the other hand *T. foenum-graceum* showed relatively better activity in inhibiting elastase but poor in protease activity in comparison to *P. corylifolia*. These variations among the most active fractions are probably due to the differences in major phytoconstituents present and their additive or synergistic response. The order of inhbition of elastase and total protease activity of plant extract was found to be *M. indica*> *T. foenum-graceum*> *P. corylifolia*. This is probably the first report on these plants active fractions. Comparable variations in inhbition of virulence factors of PAO1 have also been reported by the other workers on *Terminalia chebula*. However, the results obtained were at relatively high concentrations (Sarabhai et al. 2013). In the same way, elastase activity, total proteolytic activity and pyoverdin production of *P. aeruginosa* was decreased to varying levels (10-90%) by different plant extracts obtained from several herbs, spices and medicinal plants used in Hispanic traditional medicine (Vattem et al. 2007; Huerta et al. 2008, Abraham et al. 2012).

Pyocyanin is another important virulence factor produced under QS regulation. The role of pyocyanin in pathogenesis and especially in cystic fibrosis is well documented (Winstanley and Fothergill, 2008). In cystic fibrosis patients, the pyocyanin metabolite, along with its precursor molecule phenazine-1- carboxylic acid, leads to severe toxic effects. These two metabolites induce the apoptosis of neutrophils and damage the neutrophil-mediated host defense (Fothergill et al. 2007; Mustafa et al. 2012). Therefore, the effect of plant extracts in reducing the pyocyanin production was assessed and significant decrease ($p \leq 0.001$) was noticed. Pyocyanin

production by PAO1 was maximally (88.8%) inhibited by *M. indica* extract followed by *P. corylifolia* (86.7%) and *T. foenum-graceum* (55,6%) over control. Likewise, dose dependent decrease in pyocyanin production of PAF79 was also recorded. Similar reduction in pyocyanin was recorded from several herbs, spices and medicinal plants (Huerta et al. 2008) and in extracts of edible plants and fruits (Mustafa et al. 2010; Sarabhai et al. 2013).

We have also studied chitinase activity, a less commonly explored QS regulated trait of PAO1. Chitinase production by *P. aeruginosa* strains (PAO1 and PAF79) was reduced considerably under the influence of sub-MICs of plant extracts tested. Maximum reduction in chitinase activity of PAO1 was reduced by *P. corylifolia* (75.8%) while lowest inhibitory activity was shown by extract of *T. foenum-graceum* (47.5%). In PAF79 *T. foenum-graceum* (seed) extract demonstrated highest chitinase inhibitory activity (87%) followed by the extract of *M. indica* and *P. corylifolia*. Chitinase enzyme plays an important role in the pathogenesis of *P. aeruginosa* in cystic fibrosis patients (Manos et al. 2009). Although *P. aeruginosa* is unable to use chitin as a sole carbon source (Folders et al. 2001; Jagmann et al. 2010) but still chitinase was isolated and characterized in some clinical isolates of *P. aeruginosa* that caused initial and acute infection as compared to strains isolated from the chronic phases in the patients with cystic fibrosis suggesting that chitinase and chitinase binding proteins (CBP) may play an important role in the initial adhesion of bacteria to the lung epithelial cells (Folders et al. 2001; Tran et al. 2011). Inhibition of chitinase activity to significant levels by all three plant extracts might influence virulence and pathogenicity of *Pseudomonas* under disease conditions including cystic fibrosis.

It has been well reported that AHL-dependent QS plays a major role in the formation of a biofilm with a complex wild type architecture. In the present study, the extract of *M. indica*, *P. corylifolia* and *T. foenum-graceum* inhibited the biofilm biomass considerably ($p \leq 0.005$) in a dose-dependent manner without affecting the bacterial growth in both the strains of *P. aeruginosa*. It has also been proven that surface conditioning promotes surface adhesion and subsequent microcolony formation (Sandasi et al. 2009). Light microscopic images (Figure 47) revealed that the plant extracts efficiently reduced the number of microcolonies during the biofilm

formation of test bacterial pathogens. Therefore, it is envisaged that treatment of bacterial pathogens with plant extracts resulted in the formation of weak biofilms possibly by reducing the surface adhesion and subsequent microcolony formation. SEM analysis of *M. indica*, *P. corylifolia* and *T. foenum-graceum* extract-treated biofilms displayed disintegrated architecture than that of untreated biofilms of test bacterial pathogens (Figure 48). There is increasing evidence that AHL mediated QS plays a crucial role in the maturation of biofilms (De Kievit et al. 2001) and, therefore, inhibition of QS can possibly prevent biofilm maturation. As shown in Figure 47 and 48, biofilm formation of *P. aeruginosa* was disturbed when treated with plant extracts. Therefore, it is postulated that the biofilm inhibitor could be combined with a conventional antibiotic to efficiently control biofilm by disturbing the biofilm architecture and permitting the drug to reach the bacterial cells living inside the biofilm. The result obtained in our study are similar those with the crude extracts of *C. viminalis*, *Q. virgiana*, *T. bicolor* (Adonizio et al. 2008). Similar reduction in the biofilm of PAO1 was recorded in edible plants and fruits (Mustafa et al. 2010). Extracts of *Capparis spinosa* (Abraham et al. 2011), *Cuminum cyminum* (Abraham et al. 2012), *Lagerstroemia speciosa* (Singh et al. 2012) and *Dendrophthoe falcate* (Karthikeyan et al. 2013) have demonstrated varying levels of biofilm inhbition in *Pseudomonas aeruginosa*.

Factors closely associated with biofilm formation of *Pseudomonas aeruginosa* like swarming motility and EPS production were also evaluated in this study. EPS is essential for the development of biofilm architecture and maturation (Watnick and Kolter, 1999). Moreover, overproduction of EPS leads to alterations in the architecture of biofilm that correlates with an increased resistance of the cells to osmotic and oxidative stresses (Yildiz and Skoolnik, 1999). Furthermore, there is increasing evidence suggesting that the interference with the expression of EPS synthesis gene leads to the weakening of biofilm architecture (Bomchil et al. 2003). Since, EPS production is under the control of QS (Vu et al. 2009) interference with QS would result in the reduced synthesis of EPS or inhibit the production of EPS. Therefore it is envisaged that plant extract significantly ($p \leq 0.05$) reducing EPS will possibly also reduce the resistance level of the pathogen in sessile mode. In the present study, the total amount of EPS produced was also reduced when bacterial pathogens (PAO1 and PAF79) were treated with extract of *M. indica*, *P. corylifolia*

and *T. foenum-graceum*. Although the level of reduction in EPS is relatively less but the decrease was significant for all three active fractions. Our results find support from the reports on *Capparis spinosa* (Abraham et al. 2011), *Cuminum cyminum* (Abraham et al. 2012) who demonstrated comparable observations on the tested plant extracts.

The effects of plant extracts on swarming motility of PAO1 and PAF79 were examined. A dose dependent decrease was observed with all the three active fractions. Highest reduction in swarming motility of PAO1 and PAF79 was recorded for *M. indica* (≥70%). While similar significant inhibition (54.1-63.8%) of motility was recorded in the plants of *P. corylifolia* and *T. foenum-graceum*. QS-dependent flagellar-driven swimming motility is essential for initiation of cell/surface attachment during biofilm development (Pratt and Kotler, 1998). Because flagella could facilitate the swarming motility, inhibition in the flagellar synthesis by the extracts would facilitate the reduced swarming migration pattern. Thus, these extracts indirectly demonstrated consequences on the biofilm formation of all the target pathogens in part by interfering with its ability to reach the substratum and subsequent biofilm formation by disturbing AHL-mediated QS system. Results obtained in the present study support the findings of Niu and Gilbert (2004) in which cinnamaldehyde inhibited the swimming motility of *E. coli* by preventing the initial cell-to-surface attachment.

Perhaps, there are two likely possible mechanisms by which plant extract accomplishes the inhibition of swarming motility. The QSI compound present in these extracts might inhibit the motility either by interfering with AHL mediated cell differentiation, where the normal cells got differentiated into swarmer cells rather than inhibiting the bacterial growth as reported previously with resveratrol (Wang et al. 2006) and p-nitrophenylglycerol (Liaw et al. 2000) or by interfering with the putative AHL receptor and thereby displaces the AHL molecule. The possible interference of QSI compounds with the putative AHL receptors has already been reported with the swarming motility of *P. mirabilis* (Gram et al. 1996) and *S. marcescens* (Givskov et al. 1996) by halogenated furanones obtained from *Delisea pulchura* Since the swarming behavior essentially determines the formation of biofilm (Harshey, 2003), the reduction in these motility facilitates the reduced biofilm formation. This is also

evident from the findings of Swift et al. (2001) wherein mutant strains with altered swarming motility were shown to be defective in biofilm formation.

A number of secreted virulence factors are responsible for host tissue destruction during initiation of the infectious process by *Aeromonas* sp. Virulence factors like production of exoproteases, EPS production and the formation of biofilm is known to be regulated by *ahyRI* QS system (Williams, 2007). Sub-MICs of three plant extracts tested demonstrated dose dependent significant ($p \leq 0.005$) reduction in the total protease and EPS production by *Aeromonas hydrophila* WAF38. Highest inhibition of total protease was demonstrated by methanol fraction of *T. foenum-graceum* (71.6%) followed by *M. indica* (69.1%) and least by *P. corylifolia* (65.5%) at their respective highest tested sub-MICs. On the other hand EPS production was reduced maximally by the extract of *P. corylifolia* followed by *M. indica* and *T. foenum-graceum*. Biofilm formation was also inhibited in a concentration dependent manner by sub-MICs of extracts tested. Biofilm formed on the polystyrene plate was reduced considerably upon treatment with the plant extracts. Almost similar antibiofilm activity was demonstrated by the extracts of *M. indica* and *T. foenum-graceum* as these extracts decreased biofilm forming capability of the strain by 82% and 76.9% respectively, over control. In *A. hydrophila*, AhyR/C4-HSL-dependent quorum sensing system regulates both extracellular protease production and biofilm development (Swift et al. 1999, Lynch et al. 2002). Mutation of either *ahyI* or *ahyR* reduces or abolishes proteases activities (Lynch et al. 2002, Khajanchi et al. 2009). Findings of the investigation conducted indicate a substantial reduction in protease activity and EPS production of the test strain. Results obtained on biofilm inhibition in *A. hydrophila* are comparable to the work of Ponnusamy et al. (2009) with vanillin. The data obtained in our study for protease production and biofilm inhibition indicates that the oil is potentially acting or the AhyRI system rendering the production of C4-HSL impaired. Likewise, the effect of chestnut honey and its aqueous and methanolic extracts (0.2 g/ml) inhbited biofilm formation by 61% over control. Truchado et al. (2009) reported that both the degradation of AHLs and the inhibition of AHL production by the bacterial strain caused an attenuation of bacterial AHL-controlled virulence factors and biofilm formation.

The addition of plant extract of *M. indica*, *P. corylifolia* and *T. foenum-graceum* decreased β-galactosidase luminescence in *E. coli* MG4/pKDT17 ranging from 42-63% ($p \leq 0.005$) at their respective sub-MICs. The results of the assay demonstrate that the reduced production of AHL under the effect of sub-MICs of plant extract inhibits las-controlled transcription. A *Pseudomonas* autoinducer, AHL, is necessary in conjunction with the transcriptional activator LasR for the maximal activation of the *lasB* gene (Passador et al. 1993). Together LasR and AHL may form a critical global regulatory system for the expression of important *Pseudomonas* virulence factors. Seed et al. (1995) have shown that expression correlates with the amount of exogenous synthetic autoinducer until a saturating concentration is reached. The dependence of *lasB-lacZ* expression on autoinducer concentration was previously shown by Pearson et al. (1994). The regulation is therefore sensitive to autoinducer (AHL) concentration. The findings of the assay are in agreement with the above observations as reduced β-galactosidase activity is indicative of reduced AHL levels and therefore reduced expression of *lasB* gene. Our results also find support from the observations of Adonizio et al. (2008a) in south florida plant and Singh et al. (2012) in *Lagerstroemia speciosa* fruit extract.

Plant extracts of the above three plants are rich in polyphenolic content and also showed the presence of one more compounds belonging to major classes of flavonoids, tannins, coumarins, alkaloids etc. Our preliminary studies on these extracts using IR, HPTLC, HPLC and colour tests have revealed the presence of many major classes of phytocompounds. GC-MS analysis of the plant extracts confirmed the presence of many volatile compounds. However, further isolation and identification of major anti-QS agents is needed and the role of various phytocontituents is to be determined. Many phytocompounds belonging to these classes of phytocompounds are reported to reduce QS controlled virulence factors in different strains (Kalia, 2013). For example, biofilm formation by *E. coli* was disrupted even by grapefruit juice (Furocoumarins) (Girennavar et al. 2008) and of *P. aeruginosa* by rosamarinic acid produced by the roots of *Ocimum basilicum* (Sweet Basil) (Walker et al. 2004). Phenolic plant secondary metabolites such as salicylic acid stimulate AHL-lactonase enzyme expression (Yuan et al. 2007). Flavanoids such as naringenin, kaempferol, quercetin and apigenein inhibited HAI-1 or AI-2 mediated bioluminescence in *V. harveyi* BB886 and MM32. Quercetin and naringenin were

found to inhibit biofilm formation by *V. harveyi* BB120 and *E. coli* O157:H7 (Vikram et al. 2010). Flavan-3-ol catechin, one of the flavonoids from the bark of *Combretum albiflorum* reduces the production of QS-mediated virulence factors -pyocyanin, elastase and biofilm formation by *P. aeruginosa* PAO1 (Vandeputte et al. 2010). Curcumin has also been reported to significantly reduce bioluminescence of *V. harveyi*. Alginate, EPS production, motility and biofilm formation was also reduced considerably (Abraham et al. 2013).

Thus, it can be concluded that the effect of the extracts on modulating different virulence factors in *Pseudomonas aeruginosa* suggest that the extracts are acting at different hierarchical positions in the overall QS cascade. This is also due to the possibility that the active fractions actually contain not a single compound but a group of QS interfering compounds which give them broad spectrum capabilities to modulate the QS circuit at multiple levels mediated by different AHL molecules (Huerta et al. 2008). However, further isolation and purification of active compounds is needed to study actual mode of action.

6.4. Effect of essential oil and pure compounds on QS regulated virulence factors *Pseudomonas aeruginosa* and *Aeromonas hydrophila*

Essential oils and their active constituents are widely known for various biological activities and are traditionally used in treatment of ailments.including infectious diseases. In this study most active plant essential oils (clove and peppermint) were selected based on screening results against *Chromobacterium violaceum* strains. Our results show that clove oil exhibits concentration-dependent inhibitory activity on *C. violaceum* CV026. Clove oil at its highest tested sub-MIC (1.6%) significantly decreased all QS regulated virulence factors in PAO1 in the range of 65-85%. While similar inhibition of virulence factors at 3.2% was recorded for elastase, total protease, pyocyanin production, swarming motility and biofilm formation in PAF79.

Major ingredient of clove oil as revealed by GC–MS analysis is eugenol (74.32%), and other constituents identified were α-caryophyllene (4.05%), iso-caryophyllene (5.96%), caryophyllene oxide (2.41%), β-caryophyllene (4.92%), napthalene, 1,2,3,5,6,8a-hexahydro- 4,7-dimethyl-1-(1-methyl ethyl) (7.04%) and 1,6-Octadiene-ol-,3,7-dimethyl acetate (1.28%).

To acertain the role of major active constituents of clove oil, eugenol was also tested at its respective sub-MICs. It is interesting to note that eugenol significantly ($p \leq 0.001$) inhibited QS regulated virulence factors ranging from 49.1-87% in PAO1 but swarming motility was not reduced significantly at any of the tested concentrations. Similar trend of inhibition of virulence factors was also observed against the PAF79 as all the QS regulated traits were reduced significantly except for swarming motility over control. Our results are consistent with data of Zhou et al. (2013) that eugenol does not evidently inhibit *P. aeruginosa* swarming at 200 µM. *P. aeruginosa* has three QS systems, namely, *las, rhl*, and *pqs*. The *las* and the *rhl* systems are LuxI/LuxR homologues of LasI/LasR and of RhlI/RhlR, respectively (Dekimpe and Deziel 2009). *Pseudomonas aeruginosa swarming* motility is one of the QS-controlled factors and is mainly regulated by rhl system. The non-inhibition of the swarming motility only indicates that the *rhl* system has not been affected at tested concentrations. Our data show that clove oil and eugenol decreases both the elastase activity of PAO1 and the transcriptional activation of lasB in *E. coli*, which indicates that eugenol inhibits the las system as reported by Zhou et al. (2013). Similarly, clove oil and eugenol reduced both the pyocyanin production of PAO1 and PAF79 indicates that the oil and its major phytocontituent inhibit the *pqs* system. Considering the *las* and *pqs* systems regulate the expression of numerous virulence-related genes, using the oil and eugenol to inhibit these two systems would significantly decrease *P. aeruginosa* virulence. Clove oil and eugenol also inhbited virulence factors and biofilm formation in *Aeromonas hydrophila* significantly. At the highest tested sub-MIC (0.4%) clove demonstrated 57-66% decrease in the tested virulence factors. Similar reduction was also observed with sub-MICs of eugenol. The findings indicate broad spectrum quorum sensing inhibitory properties of clove oil and eugenol.

QS inhibitory potential of peppermint and its major constituent menthol were evaluated for their ability to inhibit AHL-dependent violacein production in *C. violaceum* and virulence factors such as elastase, protease, pyocyanin, EPS production and biofilm formation in PAO1 and PAF79. In this study, a dose dependent decrease ($p \leq 0.001$) of AHL mediated violacein production was recorded in *C. violaceum* CVO26. The results are in agreement with those reported for essential oils of rose, clove, chamomile oils and pine turpentine (Eris and Ulusoy, 2013).

Major ingredient of peppermint oil as revealed by GC– MS analysis is menthol (36.87%), and other constituents identified were menthone (16.44%), neoisomenthol (11.33%), isomenthone (10.47%), menthyl acetate (7.47%), 2-isopropyl-5-methylcyclohexanol (2.74%), piperitone (2.17%) and limonene (0.53%).

Peppermint oil reduced the elastase, total protease, chitinase activity, pyocyanin, EPS production, swarming motility and biofilm formation significantly ($p \leq 0.001$) ranging from 76-85.2% in PAO1. Similar reductions in the above virulence factors ranging from 54.5-88.1% was recorded for PAF79 at sub-MICs of the oil. Menthol the major active principle of peppermint demonstrated almost identical anti-virulence and anti-biofilm potential in the strains of *P. aeruginosa*. Further, peppermint oil and menthol showed promising activity against virulence factors of *A. hydrophila* WAF38. Significant reductions ($p \leq 0.001$) were recorded in all QS regulated traits tested as 52.2-80% decrease was observed at respective sub-MICs. Our findings are in agreement with the reports of Szabo et al. (2010) who reported similar inhibition of quorum sensing signals by oils of rose, geranium, lavender, rosemary. In another study Colombian essential oil particularly *Lippia alba* has been reported to posses anti-QS activity by inhibiting short-length AHLs (Jaramillo-Colorado et al. 2011). The results of our present investigation revealed the biofilm inhibiting potential of peppermint oil and its major phytoconstituent menthol against PAO1, PAF79 and WAF38 in a concentration dependent manner, as shown in figures 47 and 48. Recent studies on herbal extract of *Mentha piperita* have demonstrated 57% reduction in adhesion property of *P. aeruginosa* (Sandasi et al. 2011); the result confirms our findings on the biofilm inhibitory property of *Mentha piperita* oil and menthol. Similar broad spectrum anti-QS and anti-biofilm activity has been reported in essential oil of Marjoram (Kerekes et al. 2013).

The oils and phytocompounds tested to interfere with QS mechanisms, should be reflected in the transcription level of QS-controlling and QS-regulated genes. The effect of these agents on QS systems was therefore characterized by measuring the expression of *lasB* in *P. aeruginosa* PAO1 grown with or without these agents using β-galactosidase assay. Clove and peppermint oil along with their major constituents significantly reduced ($p \leq 0.05$) the AHL levels of PAO1 expressed in miller units which suggests that the action of the oils and their major phytoconstituents most

probably results from a combination of the reduction of the production of AHL molecule (which could be the major effect, and which is corroborated by the down regulation of the expression of the *lasB* gene) and of the capacity of the las-controlled transcription factors to perceive their cognate molecules, with a consequent reduction of the expression of QS-related genes. Our observations find support from the work of Vandeputte et al. (2010) who demonstrated that flavanones naringenin and taxifolin reduced the expression of several QS-controlled genes (i.e. *lasI, lasR, rhlI, rhlR, lasA, lasB, phzA1 and rhlA*) in *P. aeruginosa* PAO1.

The mode of action of essential oils containing phenolic compounds such as carvacrol, eugenol, thymol have the strongest anti-bacterial activity. Therefore carefully selected sub-MICs were tested in the present study for anti-QS properties. The main target of active constituents of both clove and peppermint oil is the cell wall and cytoplasmic membrane or proteins embedded in the membrane (Dorman and Deans, 2000). Cell membrane lost its integrity, and leakage of cell compounds can lead to death of the cell (Burt, 2004). However, their mode of action at sub-MICs as potential anti-QS and antibiofilm agents is because of the interference in the QS circuit at multiple points.

6.5. Anti-infective potential of the test agents in *C. elegans* nematode model

The nematode *C. elegans* has been widely used as a live animal model to study the pathogenicity of bacterial and fungal pathogens (Moy et al. 2006; Berger et al. 2007). This nematode has also been utilized as a host model for the assessment of the anti-infective potential of antibacterial and antifungal drugs (Moy et al. 2006; Berger et al. 2007). The advantage of using the *C. elegans* live animal model is that both the efficiency and the host toxicity of a particular anti-infective compound can be tested in parallel. Therefore, in this study, the *C. elegans* nematode model was used for the assessment of the anti-infective potential of the anti-QS agents identified in this study against PAO1 infection. The anti-infection potential of the sub-MIC of doxycycline and ceftazidime was assessed using a liquid killing assay of *C. elegans* by PAO1 in a 24-well microtitre plate at sub-MICs of antibiotics. Complete (100%) mortality of the *P. aeruginosa* PAO1 preinfected *C. elegans* was observed within 72 h. However, *C. elegans* preinfected with PAO1 and treated with doxycycline (4 µg/ml) and ceftazidime (0.5 µg/ml) separately displayed enhanced survival rate of 55% and 61%

respectively. Similarly, plant extracts of *M. indica*, *P. corylifolia* and *T. foenum-graceum* also displayed an enhanced survival rate of 72%, 58% and 48% respectively.

Essential oils and their phytocompounds tested also increased the survival of the nematode significantly (58-71%) in comparison to *P. aeruginosa* infected nematode. *In vivo* study clearly indicates that all the test agents interferes with the virulence factors of PAO1 leading to reduced mortality of *C. elegans* possibly by cyanide asphyxiation and paralysis. The death of the nematode by PAO1 is caused by the cyanide asphyxiation and paralysis (Gallagher and Manoil, 2001). The *hcn* operon mediates cyanide production in PAO1 and is controlled by the LasR and RhlR QS regulators (Pessi and Haas, 2000). Attenuation of virulence and nematode mortality has been shown with both Δ*lasR* (Darby et al. 1999) and Δ*hcn* (Gallagher and Manoil, 2001) strains. Thus, the results from the paralytic assay suggest that the addition of these extracts are affecting the production of cyanide either through *hcn* directly; or indirectly via the QS genes. Similar enhanced survival of the nematode (*C. elegans*) after treatment with garlic extract on wild type *P. aeruginosa* reduced the mortality of worms to 40% and 5% respectively (Rasmussen et al. 2005b). Aqueous extracts of three south florida plants: *Conocarpus erectus*, *Callistemon viminalis* and *Bucida buceras* were examined for their effects on *P. aeruginosa killing* of nematode *C. elegans*. The tested plant extracts prevented mortality via gut infection in almost 60% of the worms and reduced death from toxin by 50-90% (Adonizio et al. 2008b). Addition of purified PvdQ acylase to the worms infected with *P. aeruginosa* PAO1 resulted in reduced pathogenicity and increased life span of the worms (Papaioannou et al. 2009). Enhanced survival (66%) of PAO1-preinfected *Caenorhabditis elegans* was observed after treatment with 2,5-piperazinedione (Musthafa et al. 2012). Recently, ellagic acid derivatives demonstrated enhanced survival of *C. elegans* over untreated control with lethal time increasing from 24 to 72 h (Sarabhai et al. 2013).

6.7. Highlights of the investigation

The major findings of this investigation may be highlighted as follows:

1. *Pseudomonas aeruginosa* and other Gram negative bacteria isolated from clinical and hospital environment are resistant to commonly used antibiotics and have biofilm forming ability and could produce one or more quorum

sensing signals (AHL) molecules. Both clinical and environmental isolates have active QS systems.

2. Amongst the antibiotics screened, quorum sensing inhibition activity was found in Doxycycline followed by Ceftazidime, Ciprofloxacin, Erythromycin and Kanamycin in *Chromobacterium violaceum* based assay system.

3. Among the 37 medicinal plant extract tested against *Chromobacterium violaceum* biosensor strains, 10 extracts demonstrated QS interference of varying degree. The extracts of *Mangifera indica* (leaf), *Plumbago zeylanica* (root), *Camelia sinensis* (leaf), *Psoralea corylifolia* (seed), *Terminalia chebula* (fruit), *Cuminum cymimum* (fruit), *Holarrhena antidysenterica* (bark), *Delonix regia* (flower) and *Lawsonia inermis* decreased the violacein production while *Trigonella foenum-graceum* (seed) increased the production of violacein in the biosensor strains.

4. Fraction based activity determination of three plants (*Mangifera indica, Psoralea corylifolia* and *Trigonella foenum-graceum*) indicated that promising quorum sensing interference activity is located in methanol fraction.

5. Of the 21 essential oils tested, oils of clove, cinnamon, lavender and peppermint showed QS inhibition in the strains of *Chromobacterium violaceum*. Highest activity was detected in clove and peppermint oils and their major constituents eugenol and menthol, respectively.

6. QS regulated virulence factors in *Pseudomonas aeruginosa* and *Aeromonas hydrophila* were inhibited in a concentration dependent manner by antibiotics Doxycycline and ceftazidime. Similar reduction in the biofilm and its associated factors like exopolysaccharide (EPS) production and swarming motility was also significantly inhibited.

7. Methanol extract of three plants (*Mangifera indica, Psoralea corylifolia* and *Trigonella foenum-graceum*) significantly inhibited virulence factors like elastase, total protease, chtinase, pyocyanin production, EPS, swarming motility and biofilm formation to varying levels in a concentration dependent manner.

8. Clove and peppermint oil inhibited biofilm and QS regulated virulence traits of PAO1, PAF79 and *A. hyrophila* WAF38. The efficacy of the oils was found mainly attributed to their essential active constituents, eugenol and menthol.
9. β-galactosidase assay revealed that all the test agents significantly reduced AHL level sat respective sub-MICs. Reduced AHL levels are indicative of impaired *las*-controlled transcription, therefore reduced expression of *lasB* gene.
10. The efficacy of anti-QS active antibiotics, plant extracts, essential oils and phytocompounds in *C. elegans* model highlights their therapeutic potential in combating PAO1 infection.
11. Preliminary phytochemical analysis of active plant extracts indicated that these extracts are rich in phenolic content and an array of compounds are present as revealed by colour test, IR, HPTLC and UPLC analysis. GC-MS analysis of the plant extracts revealed the presence of variety of volatile compounds. Further isolation and identification of active compounds is needed to uncover the therapeutic potential of the extracts.
12. GC-MS analysis of clove and peppermint oil reveals the presence of many compounds including major compounds such as eugenol and methanol in the above oils.

6.8. Future prospects

The present investigation clearly reveals that anti-QS property is present in many Indian medicinal plants and their essential oils. However, many of the extracts/oil could also inhibit growth of bacteria at higher concentrations. Such extracts are also known for other biological activities. Further, careful investigation in identifying the key compounds responsible for such broad spectrum antivirulence and antibiofilm activity should be isolated and role of other interacting molecules may be explored. Future research in this direction requires understanding the actual mechanism of QS inhibition at molecular level. On the other hand standardized active plant extracts/essential oils and phytocompounds (eugenol, menthol) could be effectively attempted in developing modern herbal preparations for effective evaluation in animal model system for their therapeutic applications. Combinational approach of anti-QS agents from plants with antibiotics needs to be explored for possible strategy to

combat pathogens. It is interesting to note that most of the anti-QS agents effective in CVO26, PAO1, PAF79 and WAF38 effectively inhibited biofilm formation. Therefore, these agents might also be effective in inhibiting or disrupting biofilm formation in other bacteria and may be become an attractive agent for dealing with biofilm based persistent infections or in improving efficacy of old antibiotics by tolerance levels.

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Annexure

Annexure 1: Details of the patients and the sources of clinical samples

S.No	Name	Stay	Status	CADS No.	Age	Sex	Occupation	Address	Type of sample
1	Vinod Kumar	21	discharged	26840/09	28	M	shop keeper	Akbarabad, Aligarh	Blood
2	Sabira Khan	12	discharged	25789/09	46	F	house wife	Shahjahanpur	Pus
3	Panna Lal	2	discharged	12992/09	65	M	servent	Atrauli, Aligarh	Sputum
4	Raisa Begum	28	discharged	291396/09	36	F	house wife	Jamalpur, Aligarh	UTI
5	Hari Om	22	discharged	24366/09	50	M	service	Pilibhit, Bareily	B-fluid
6	Rama Shankar	15	discharged	29577/09	41	M	retired	Sunder nagar, Aligarh	Pus
7	Bilal	28	discharged	29902/09	20	M	attendent	Bareilly	Cathater
8	Anisa Begum	34	discharged	30045/09	29	F	house wife	Bhamola, Aligarh	Sputum
9	Kishan Kumar	15	discharged	27977/09	45	M	labour	Hathras	UTI
10	Rakesh	9	discharged	30644/09	40	M	servent	Aligarh	Pus

Annexure 2a: *Pseudomonas aeruginosa* strain PAF14 (KF813066) 16S ribosomal RNA gene, partial sequence

GGGGAAGCGTTTATCGGATTCTGGGCGTAAGCGCGCGTAGGTGGTTCAGCAAGTTGGATGTGAAATCCC
CGGGCTCAACCTGGGAACTGCATCCAAAACTACTGAGCTAGAGTACGGTAGAGGGTGGTGGAATTTCCT
GTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGATACT
GACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAACGAT
GTCGACTAGCCGTTGGGATCCTTGAGATCTTAGTGGCGCAGCTAACGCGATAAGTCGACCGCCTGGGGA
GTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGTTTA
ATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGCAGAGAACTTTCCAGAGATGGATGGGTGC
CTTCGGGAACTCTGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAAGTC
CCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACCTCGGGTGGGCACTCTAAGGAGACTGCCGGT
GACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACGTGC
TACAATGGTCGGTACAAAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAGTCC
GGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGAATCAGAATGTCACGGT
GAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGGAGTGGGTTGCTCCAGAAGTAGCT
AGTCTAACCGCAAGGGGGACGGTTACCACGGAGTGATTCATGACTGGGTGATAGAAGAAAAAGGCCAAA
AAAAAAAAAACAAAAAAAAAAAGAAAAAAAGAGGGGGGTGT

Annexure 2b: *Pseudomonas aeruginosa* strain PAF79 (JX424425) 16S ribosomal RNA gene, partial sequence

GGGGAAAACGTTAATCGGATTACTGGGGCGTAAGCGCGCGTAGGTGGTTCAGCAAGTTGGATGTGAAAT
CCCCGGGCTCAACCTGGGAACTGCATCCAAAACTACTGAGCTAGAGTACGGTAGAGGGTGGTGGAATTT
CCTGTGTAGCGGTGAAATGCGTAGATATAGGAAGGAACACCAGTGGCGAAGGCGACCACCTGGACTGAT
ACTGACACTGAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAAAC
GATGTCGACTAGCCGTTGGGATCCTTGAGATCTTAGTGGCGCAGCTAACGCGATAAGTCGACCGCCTGG
GGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTGGT
TTAATTCGAAGCAACGCGAAGAACCTTACCTGGCCTTGACATGCTGAGAACTTTCCAGAGATGGATTGG
TGCCTTCGGGAACTCAGACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTTAA
GTCCCGTAACGAGCGCAACCCTTGTCCTTAGTTACCAGCACCTCGGGTGGGCACTCTAAGGAGACTGCC
GGTGACAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACACACG
TGCTACAATGGTCGGTACAAAGGGTTGCCAAGCCGCGAGGTGGAGCTAATCCCATAAAACCGATCGTAG
TCCGGATCGCAGTCTGCAACTCGACTGCGTGAAGTCGGAATCGCTAGTAATCGTGAATCAGAATGTCAC
GGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGGAGTGGGTTGCTCCAGAAGTA
GCTAGTCTAACCGCAAGGGGGACGGTTACCACGGAGTGATTCATGATGGGGGATAACAAAAAACCCCCC
CAAAAAAAAAAAAAAAAATCCCCCCCCGGAAGGGGGGGGG

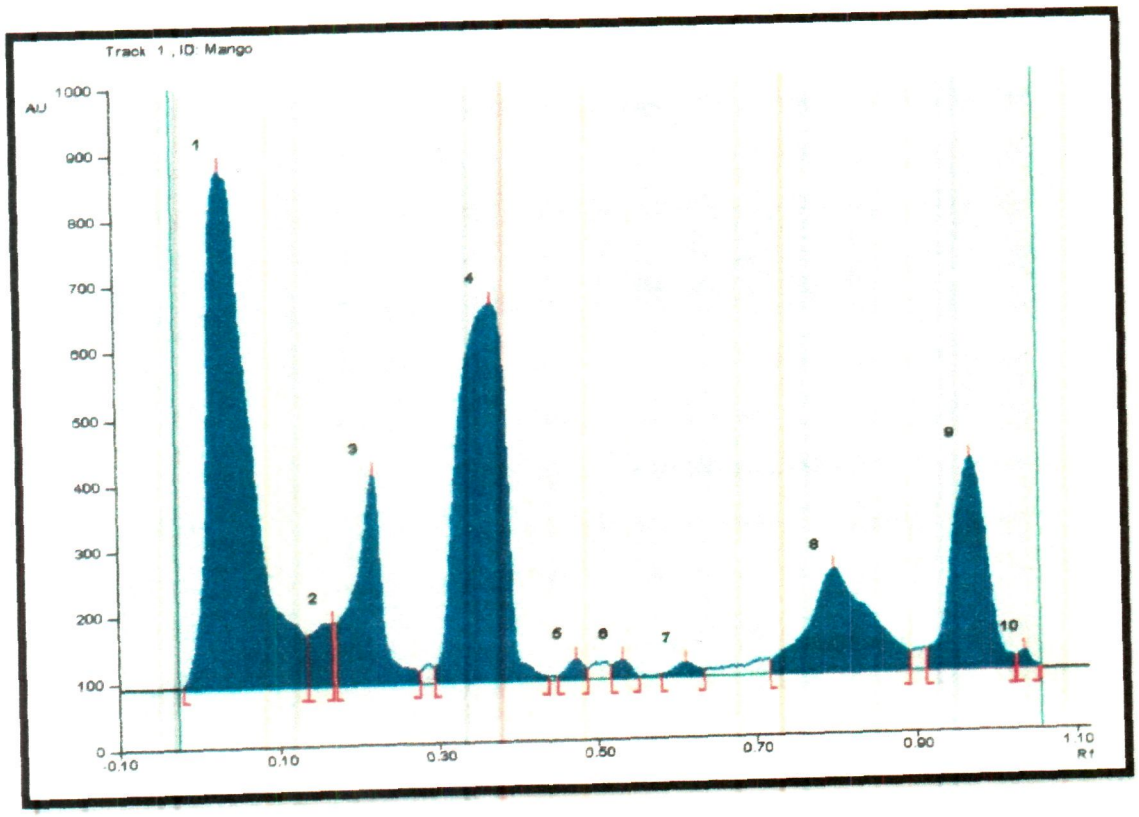
Annexure 3a: *Aeromonas hydrophila* strain WAF38 (JX416386) 16S ribosomal RNA gene, partial sequence

GGGGGGCACGTTTATCGGAAATTACTGGGCGTAAAGCGCACGCAGGCGGTTGGATAAGTTAGATGTGAA
AGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCCAGCTAGAGTCTTGTAGAGGGGGGTAGAAT
TCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCCTGGACAA
AGACTGACGCTCAGGTGCGAAAGCGTGGGGAGCAAACAGGATTAGATACCCTGGTAGTCCACGCCGTAA
ACGATGTCGATTTGGAGGCTGTGTCCTTGAGACGTGGCTTCCGGAGCTAACGCGTTAAATCGACCGCCT
GGGGAGTACGGCCGCAAGGTTAAAACTCAAATGAATTGACGGGGGCCCGCACAAGCGGTGGAGCATGTG
GTTTAATTCGATGCAACGCGAAGAACCTTACCTGGCCTTGACATGTCTGGAATCCTGCAGAGATGCGGG
AGTGCCTTCGGGAATCAGAACACAGGTGCTGCATGGCTGTCGTCAGCTCGTGTCGTGAGATGTTGGGTT
AAGTCCCGCAACGAGCGCAACCCCTGTCCTTTGTTGCCAGCACGTAATGGTGGGAACTCAAGGGAGACT
GCCGGTGATAAACCGGAGGAAGGTGGGGATGACGTCAAGTCATCATGGCCCTTACGGCCAGGGCTACAC
ACGTGCTACAATGGCGCGTACAGAGGGCTGCAAGCTAGCGATAGTGAGCGAATCCCAAAAAGCGCGTCG
TAGTCCGGATCGGAGTCTGCAACTCGACTCCGTGAAGTCGGAATCGCTAGTAATCGCAAATCAGAATGT
TGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCGTCACACCATGGGGAGTGGGTTGCACCAGAA
GTAGATAGCTTAACCTTCGGGAGGGCGTTTACCACGGTGTGATTATGATGGGGTGAT

Annexure 3a: *Aeromonas aquariorum* strain WAF47 (KF813065) 16S ribosomal RNA gene, partial sequence

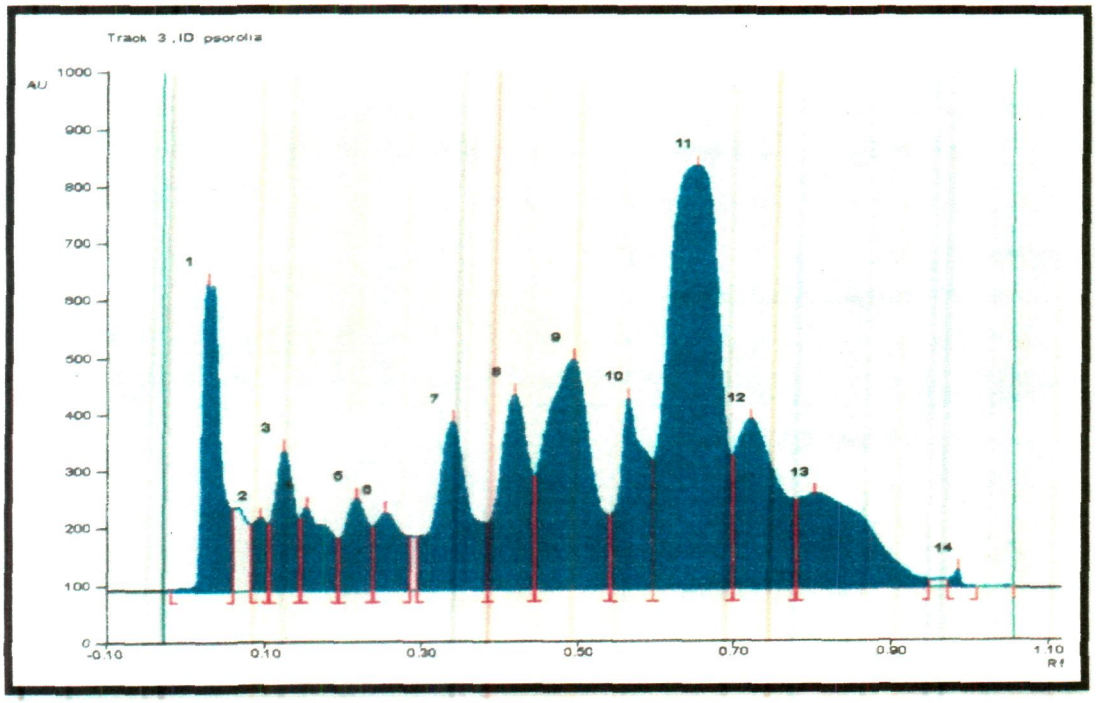
TTCTTTTTCGGGGGCACTTATATATAGGGTTTTTTTTCTGCTCAGATTGAACGCTGGCGGCAGGCCTAA
CACATGCAAGTCGAGCGGCAGCGGGAAAGTAGCTTGCTACTTTTGCCGGCGAGCGGCGGACGGGTGAGT
AATGCCTGGGAAATTGCCCAGTCGAGGGGGATAACAGTTGGAAACGACTGCTAATACCGCATACGCCCT
ACGGGGGAAAGCAGGGGACCTTCGGGCCTTGCGCGATTGGATATGCCCAGGTGGGATTAGCTAGTTGGT
GAGGTAATGGCTCACCAAGGCGACGATCCCTAGCTGGTCTGAGAGGATGATCAGCCACACTGGAACTGA
GACACGGTCCAGACTCCTACGGGAGGCAGCAGTGGGGAATATTGCACAATGGGGGAAACCCTGATGCAG
CCATGCCGCGTGTGTGAAGAAGGCCTTCGGGTTGTAAAGCACTTTCAGCGAGGAGGAAAGGTCAGTAGC
TAATATCTGCTGACTGTGACGTTACTCGCAGAAGAAGCACCGGCTAACTCCGTGCCAGCAGCCGCGGTA
ATACGGAGGGTGCAAGCGTTAATCGGAATTACTGGGCGTAAAGCGCACGCAGGCGGTTGGATAAGTTAG
ATGTGAAAGCCCCGGGCTCAACCTGGGAATTGCATTTAAAACTGTCCAGCTAGAGTCTTGTAGAGGGGG
GTAGAATTCCAGGTGTAGCGGTGAAATGCGTAGAGATCTGGAGGAATACCGGTGGCGAAGGCGGCCCCC
TGGACAAAGACTGACGCTCAGTGCGAAAGAACGCCC

Track	Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	1	-0.02 Rf	4.3 AU	0.04 Rf	83.7 AU	3.34 %	0.14 Rf	5.8 AU	3210.8 AU	5.66 %	unknown *
1	2	0.14 Rf	76.2 AU	0.17 Rf	94.0 AU	4.00 %	0.17 Rf	3.8 AU	2371.9 AU	2.16 %	unknown *
1	3	0.17 Rf	92.7 AU	0.22 Rf	19.4 AU	3.59 %	0.28 Rf	3.0 AU	5089.7 AU	9.18 %	unknown *
1	4	0.30 Rf	24.6 AU	0.38 Rf	71.4 AU	4.31 %	0.44 Rf	5.1 AU	1898.9 AU	9.01 %	unknown *
1	5	0.45 Rf	3.6 AU	0.48 Rf	31.2 AU	1.33 %	0.49 Rf	3.5 AU	666.7 AU	0.61 %	unknown *
1	6	0.52 Rf	20.2 AU	0.54 Rf	29.0 AU	1.23 %	0.56 Rf	3.7 AU	556.7 AU	0.51 %	unknown *
1	7	0.58 Rf	3.8 AU	0.61 Rf	20.6 AU	0.88 %	0.64 Rf	3.5 AU	592.9 AU	0.54 %	unknown *
1	8	0.72 Rf	20.8 AU	0.80 Rf	55.9 AU	6.63 %	0.90 Rf	3.3 AU	1300.4 AU	0.28 %	unknown *
1	9	0.92 Rf	31.7 AU	0.98 Rf	20.9 AU	3.65 %	1.03 Rf	7.7 AU	2944.6 AU	1.77 %	unknown *
1	10	1.03 Rf	18.0 AU	1.04 Rf	24.3 AU	1.04 %	1.06 Rf	3.9 AU	332.3 AU	0.30 %	unknown *



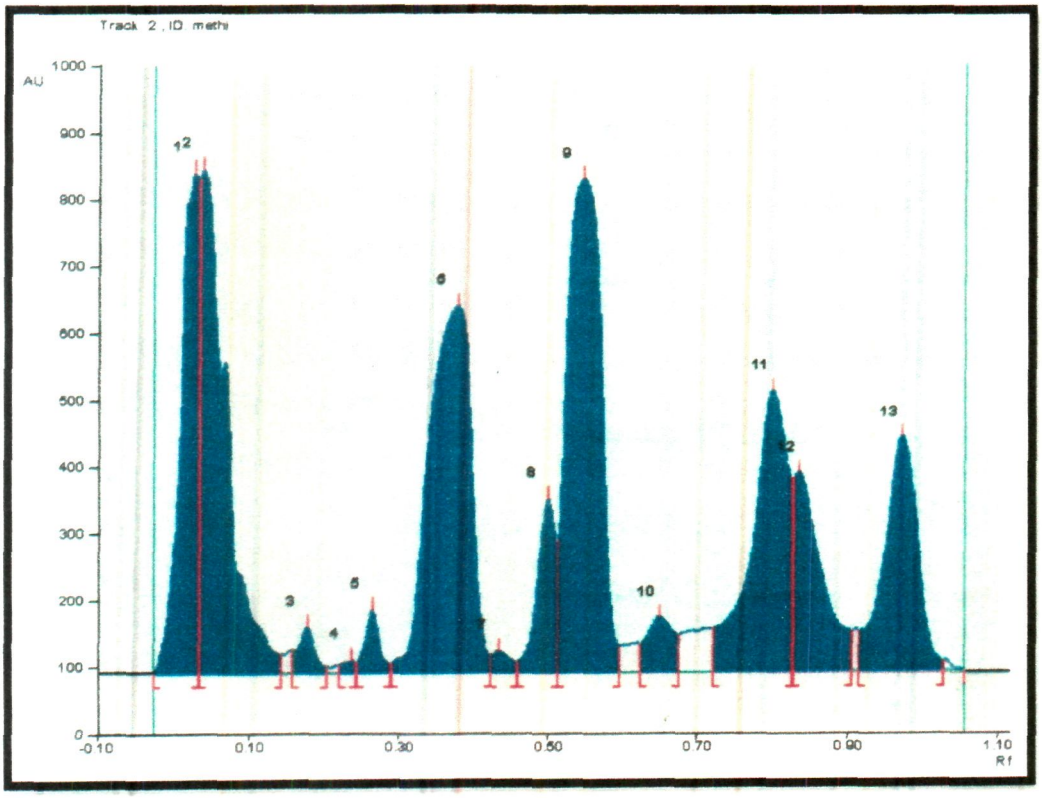
Annexure 4: HPTLC profile and chromatogram of *Mangifera indica* (leaf) methanol extract

Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
2	1	-0.02 Rf	2.0 AU	0.03 Rf	49.4 AU	6.92 %	0.04 Rf	3.3 AU	7098.1 AU	0.52 %	unknown *
2	2	0.04 Rf	39.8 AU	0.04 Rf	56.0 AU	7.07 %	0.15 Rf	7.7 AU	5271.3 AU	5.54 %	unknown *
2	3	0.16 Rf	33.7 AU	0.18 Rf	89.1 AU	1.56 %	0.21 Rf	3.7 AU	1517.0 AU	0.93 %	unknown *
2	4	0.22 Rf	11.8 AU	0.24 Rf	19.6 AU	0.44 %	0.25 Rf	3.5 AU	326.4 AU	0.20 %	unknown *
2	5	0.25 Rf	16.9 AU	0.27 Rf	95.8 AU	2.16 %	0.29 Rf	1.0 AU	1718.4 AU	1.06 %	unknown *
2	6	0.29 Rf	14.3 AU	0.38 Rf	51.0 AU	2.44 %	0.43 Rf	5.8 AU	3819.9 AU	8.34 %	unknown *
2	7	0.43 Rf	25.9 AU	0.44 Rf	32.9 AU	0.74 %	0.46 Rf	3.8 AU	752.6 AU	0.46 %	unknown *
2	8	0.46 Rf	16.4 AU	0.50 Rf	59.1 AU	5.85 %	0.52 Rf	3.5 AU	5982.9 AU	3.68 %	unknown *
2	9	0.52 Rf	99.5 AU	0.55 Rf	38.9 AU	6.68 %	0.60 Rf	3.2 AU	2269.2 AU	9.85 %	unknown *
2	10	0.63 Rf	42.4 AU	0.65 Rf	84.2 AU	1.90 %	0.68 Rf	7.0 AU	2883.3 AU	1.77 %	unknown *
2	11	0.73 Rf	96.1 AU	0.81 Rf	22.4 AU	9.54 %	0.83 Rf	3.8 AU	3271.7 AU	1.85 %	unknown *
2	12	0.83 Rf	91.1 AU	0.84 Rf	99.2 AU	6.75 %	0.91 Rf	3.7 AU	6694.0 AU	6.58 %	unknown *
2	13	0.92 Rf	81.8 AU	0.98 Rf	52.2 AU	7.95 %	1.03 Rf	5.6 AU	1972.3 AU	9.21 %	unknown *

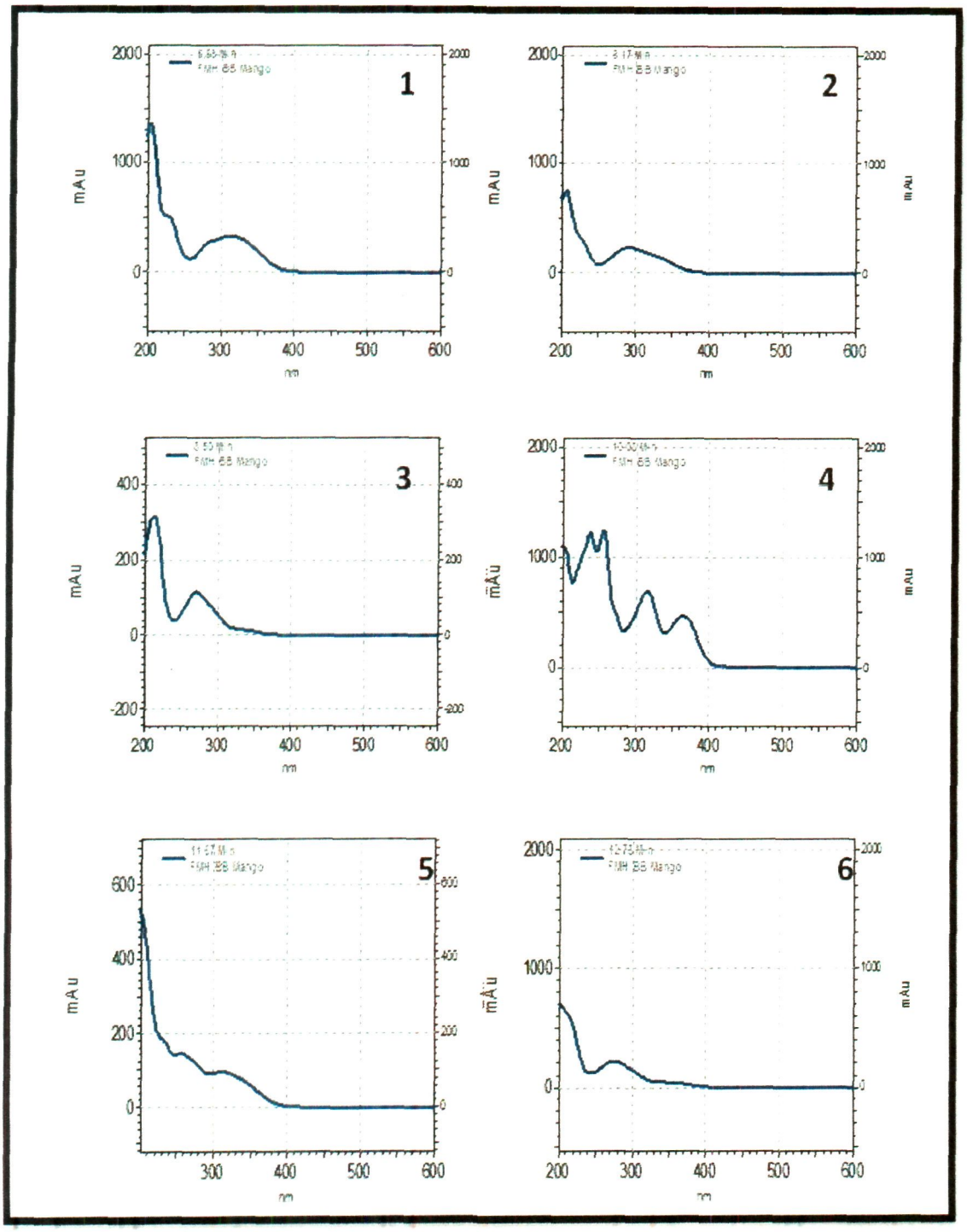


Annexure 5: HPTLC profile and chromatogram of *Psoralea corylifolia* (seed) methanol extract.

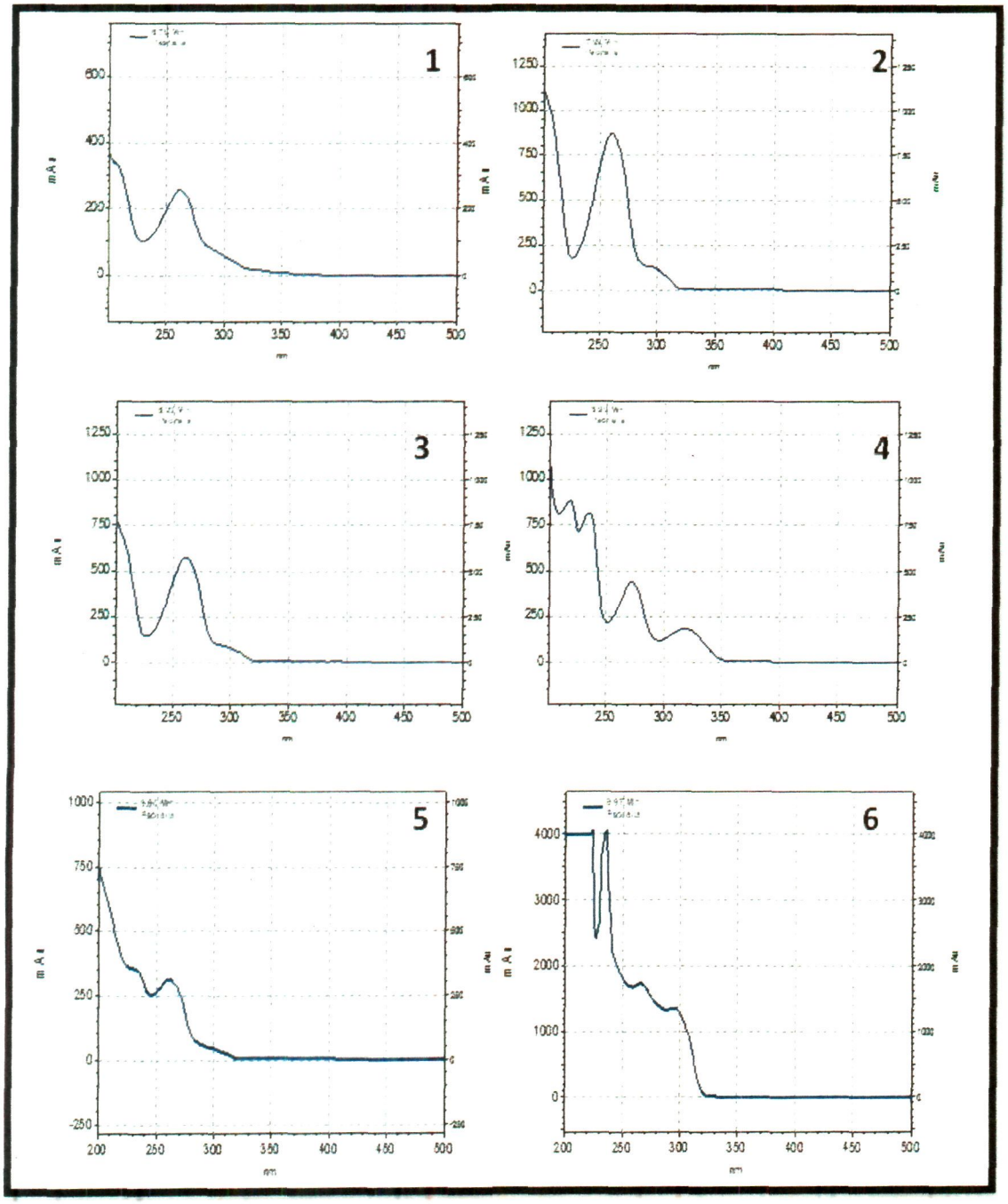
Peak	Start Position	Start Height	Max Position	Max Height	Max %	End Position	End Height	Area	Area %	Assigned substance
1	-0.01 Rf	3.2 AU	0.03 Rf	39.4 AU	3.59 %	0.06 Rf	3.8 AU	1549.2 AU	6.95 %	unknown *
2	0.09 Rf	15.2 AU	0.10 Rf	26.5 AU	3.19 %	0.11 Rf	5.5 AU	2296.6 AU	1.38 %	unknown *
3	0.11 Rf	16.4 AU	0.13 Rf	45.1 AU	6.18 %	0.15 Rf	4.9 AU	5964.3 AU	3.59 %	unknown *
4	0.15 Rf	26.5 AU	0.16 Rf	45.1 AU	3.66 %	0.20 Rf	0.6 AU	4686.8 AU	2.82 %	unknown *
5	0.20 Rf	91.1 AU	0.22 Rf	60.5 AU	4.04 %	0.24 Rf	0.0 AU	4614.8 AU	2.78 %	unknown *
6	0.24 Rf	10.2 AU	0.26 Rf	35.3 AU	3.41 %	0.29 Rf	1.3 AU	4498.5 AU	2.71 %	unknown *
7	0.30 Rf	93.5 AU	0.34 Rf	95.7 AU	7.45 %	0.39 Rf	6.4 AU	2359.0 AU	7.44 %	unknown *
8	0.39 Rf	16.8 AU	0.42 Rf	42.9 AU	8.64 %	0.45 Rf	8.1 AU	1830.5 AU	7.12 %	unknown *
9	0.45 Rf	98.1 AU	0.50 Rf	05.0 AU	0.21 %	0.55 Rf	8.9 AU	2539.3 AU	3.56 %	unknown *
10	0.55 Rf	29.3 AU	0.57 Rf	35.8 AU	8.46 %	0.60 Rf	5.4 AU	0501.6 AU	6.32 %	unknown *
11	0.60 Rf	26.2 AU	0.66 Rf	44.5 AU	8.76 %	0.70 Rf	1.1 AU	5610.7 AU	7.45 %	unknown *
12	0.70 Rf	32.1 AU	0.73 Rf	97.3 AU	7.49 %	0.78 Rf	4.3 AU	5297.1 AU	9.21 %	unknown *
13	0.79 Rf	54.6 AU	0.81 Rf	65.2 AU	4.16 %	0.95 Rf	3.5 AU	4094.9 AU	8.48 %	unknown *
14	0.98 Rf	13.2 AU	0.99 Rf	30.4 AU	0.77 %	1.01 Rf	0.8 AU	332.0 AU	0.20 %	unknown *



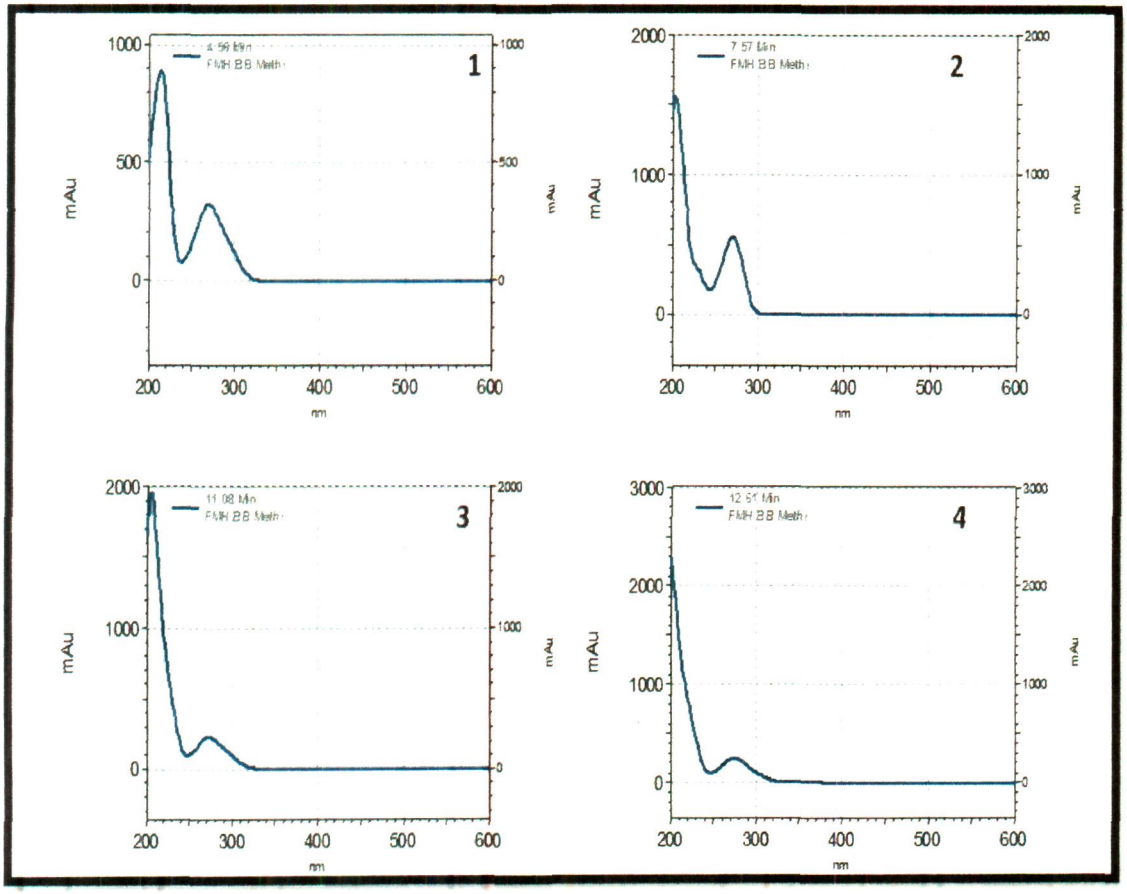
Annexure 6: HPTLC profile and chromatogram of *Trigonella foenum-graceum* (seed) methanol extract.



Annexure 7: UPLC analysis of peaks of *Mangifera indica* (leaf) extract by photo-diode array detector



Annexure 8: UPLC analysis of peaks of *Psoralea corylifolia* (seed) extract by photo-diode array detector.



Annexure 9: UPLC analysis of peaks of *Trigonella foenum-graceum* (seed) extract by photo-diode array detector.

Summary

In many pathogenic gram negative bacteria the production of virulence factors is triggered in a population density-dependent manner through quorum sensing (QS), a cell-to cell communication mechanism that enables bacteria to coordinate virulence factor production by means of the synthesis, release and perception of small diffusible molecules called acyl homoserine lactone (AHLs). Opportunistic pathogen *Pseudomonas aeruginosa*, has two main QS systems (lasI/R and rhlI/R), responsible for the synthesis and perception of the acylhomoserine lactones (AHLs) 3-oxo-C12-HSL and C4-HSL that control the expression of an arsenal of virulence factors and biofilm formation. It has also been found that bacteria living in the biofilm mode of growth are often up to 1000 times more resistant to antibiotic than their planktonic counterparts. Antibiotic resistance and biofilm are the major obstacles in treatment of bacterial infections.

Current therapies rely on antibiotic treatments that result in death of invading bacteria leading to development of resistance. In the new millennium we are encountering an alarming increase in multi-resistant bacteria, and as a result infections that were once treatable have become non-treatable. This calls for development of alternative treatment strategies and one appealing approach, is the anti-pathogenic drug principle, to selectively block the control apparatus of virulence and pathogenic traits of the bacteria. By doing so, the infecting bacteria may fail to adapt to the host environment and establish an infection.

Medicinal and dietary plants are known to offer a large and attractive phytochemical repertoire for the discovery of novel microbial disease control agents. Considering the rich diversity of traditionally used medicinal plants and essential oil in a number of ailments/infectious diseases in traditional system of medicine. It is expected that systemic screening and evaluation of Indian medicinal plants and other natural products will hopefully provide new bioactivity relevant to anti-pathogenic drug principle as majority of Indian medicinal plants are not yet systematically screened and evaluated for QS interference of pathogenic bacteria.

Therefore, screening of plant and its derived compounds may facilitate the discovery of compounds that attenuate bacterial pathogenesis by interfering with QS systems and render pathogenic bacteria non-virulent without affecting their viability. Considering the need of new anti-infective drugs and treatment strategies and lack of

concerted efforts to screen rich diversity of Indian medicinal plants, the present study was planned with the following aims and objectives:

Objectives:

1. Isolation, characterization and detection of quorum sensing signal molecules in *Pseudomonas aeruginosa* and other Gram negative bacteria.
2. Screening of natural products (antibiotics, plant extracts and essential oils) for their quorum sensing interference activity against *Chromobacterium violaceum* biosensor strains.
3. Evaluation of active antibiotics on quorum sensing regulated virulence factors and biofilm formation in the strains of *Pseudomonas aeruginosa* and *Aeromonas hydrophila*.
4. Fraction based activity of active plant extracts and active essential oils on quorum sensing regulated virulence factors and biofilm of test bacterial strains.
5. To evaluate the therapeutic potential of active extracts, essential oils and phytocompounds in *Caenorhabditis elegans* nematode model.
6. Phytochemical analysis of selected plant extracts and essential oils.

The work done on the basis of above objectives is briefly summarized below.

Isolation, biochemical characteristics and AHL characterization among bacterial isolates

A total of 55 bacterial isolates belonging to genera (*Pseudomonas*, *Aeromonas* sp. and *Klebsiella*) were isolated from clinical and environmental sources on their respeective media. The isolated bacteria were subjected to biochemical characterization and tentatively identified. Few promising bacterial isolates were selected for 16S rRNA gene based identification. Web-based search and phylogenetic analysis showed that PAF14, PAF79, WAF38 and WAF47 were identified as strains of *Pseudomonas aeruginosa, Aeromonas hydrophila* and *Aeromonas aquariorum* respectively. All strains shared 99% similarity in the BLAST search.

These isolates were tested for antibiotic sensitivity of the test isolates against 17 antibiotics. Maximum resistance against aztreonam (60%), followed by

amoxicillin, erythromycin (47.2% each), nitrofurantoin, polymixin-B (38.1% each), clinadamycin (36.3%), cefuroxime (32.7%), kanamycin (27.2%), doxycycline (25.4), ceftriaxone (23.6%), cephoxitin (20%), sparfloxacin (16.3%), gentamicin (12.7%), cefpirome (10.9%), ciprofloxacin (9%), imepenem (7.2%) and least resistance was observed against gatifloxacin (05.4%). Most of the isolates showed resistance to multiple antibiotics. Isolates showed varied pattern of resistance ranging from combination of 3 to 10 different antibiotics. The above isolates were further subjected to detection of quorum sensing signal (AHL) production.

A total of 29 isolates were found positive for short length (AHL) production with CV026 biosensor strain. In *Agrobacterium tumefaciens* A136 strain all the 29 isolates were also detected positive for AHL production. Further, TLC analysis revealed that total of seven *Pseudomonas* isolates showed the presence of C6-HSL while C4-HSL was also detected in all the isolates of *Pseudomonas* producing AHL. Two isolates of *Aeromonas* (WAF38 and WAF47) produced C6-HSL. Since *C. violaceum* CV026 cannot detect AHLs with longer acyl side chains, broad range biosensor, *A. tumefaciens* A136 was employed thereafter for TLC. Presence of 3-oxo-C12-HSL was detected in 9 *Pseudomonas* isolates. Unidentified AHL were also detected in *Pseudomonas* isolates PAF2, PAF14, PAF26, PAF79 and WAF47. In selected isolates, the partially purified AHLs along with standard AHLs were subjected to mass spectroscopic (MS) analysis to confirm the identity of C4-HSL, C6-HSL and 3-oxo-C12-HSL in above isolates.

All the 29 AHL producing isolates were tested for the presence of quorum sensing regulated virulence factors namely elastase activity, total protease, pyocyanin production, chitinase activity, exopolysaccharide (EPS) production, swarming motility and biofilm formation. Among the *Pseudomonas* isolates, PAF79 produced all the tested virulence factors strongly. Remaining isolates were also detected to produce varying levels of different virulence factors tested. Among the *Aeromonas* isolates WAF38 showed significant level of production of total protease, EPS and biofilm.

β-galactosidase activity in AHL producing bacterial isolates

Quantitative induction of β-galactosidase was also estimated in all the AHL producing strains of *Pseudomonas*. The control strain of *Pseudomonas aeruginosa* PAO1

produced 590±21 miller units (MU) of AHL while production of AHLs was found to be variable among the isolates. AHL levels produced by the selected isolates ranged from 124-499 miller units. Based on the highest level of quorum-sensing signal production in miller units and on the basis of the virulence factor production, strains *P. aeruginosa* PAF79 and *A. hydrophila* WAF38 were selected for QS interference activity.

Screening for quorum sensing interference by antibiotics, plant extracts and essential oils

On the basis of isolation and characterization of bacterial isolates for quorum sensing regulated functions two isolates *Pseudomonas aeruginosa* PAF79 and *Aeromonas hydrophila* WAF38 and standard strains *Chromobacterium violaceum* ATCC 12472, *Chromobacterium violaceum* CVO26, *Pseudomonas aeruginosa* PAO1 were used to screen antibiotics, plant extracts and essential oils/phytocompounds.

Screening of antibiotics for their violacein inhibitory activity

Among 33 antibiotics tested six antibiotics namely ceftazidime, ciprofloxacin, doxycycline, erythromycin, kanamycin and tobramycin showed varying levels of pigment inhibition in the test strain (CV12472 and CVO26). Doxycycline and ceftazidime produced the highest zone of pigment inhibition in both the biosensor strains.

Screening of Indian medicinal plants for quorum sensing interference activity

A total of 37 medicinal plants extracted in methanol were screened against *Chromobacterium violaceum* strains (CV12472 and CVO26). In CV12472 test system *Mangifera indica* (leaf) and *Plumbago zeylanica* (root) gave highest zone of pigment inhibition followed by *Camelia sinensis* (leaf), *Psoralea corylifolia* (seed), *Terminalia chebula* (fruit), *Cuminum cymimum* (fruit), *Holarrhena antidysenterica* (bark), *Delonix regia* (flower) and least by *Lawsonia inermis* (leaf). It is interesting to note that methanolic extract of *Trigonella foenum-graceum* seed enhanced the pigment produced by the strain and a dark zone of purple pigment was observed.

Out of the 9 plant extract reducing violacein in CV12472 only the extracts of *M. indica* and *P. corylifolia* inhibited the significant pigment production in CVO26 strain. While, the extract of *Trigonella foenum-graceum* (seed) enhanced the pigment

production in CVO26 also. On the basis of strong anti-QS activity in both strains, extracts of *M. indica* (leaf) and *P. corylifolia* (seed) along with *T. foenum-graceum* (seed) extract were subjected to fractionation based activity determination.

Fraction based violacein inhibition/interference activity of plant extracts

Different fractions of *Mangifera indica* (leaves), *Psoralea corylifolia* (seed) and *Trigonella foenumgraecum* (seed) obtained in petroleum ether, benzene, ethyl acetate, acetone and methanol were tested for their QS modulatory activity at varying concentrations against *Chromobacterium violaceum* 12472 (CV12472) strain. The study revealed that the methanol fraction in all the three plants was most active in interfering with the violacein production in CV12472. Methanol fractions of *Mangifera indica* (leaves), *Psoralea corylifolia* (seed) showed highest pigment inhibtitory activity at respective tested concentrations. While *Trigonella foenumgraecum* (Seed) extract demonstrated pigment enhancement in CV12472 strain. Other fractions exhibited antibacterial activity or non significant inhibition of pigment.

Screening of essential oils for violacein inhibition in *Chromobacterium violaceum*

In addition to the above plant extracts, 21 essential oils were also screened for potential anti-QS property. QS regulated violacein production was inhibited by four essential oils at tested concentrations. Highest activity was recorded in clove oil followed by peppermint oil, lavender and least in cinnamon. Clove oil and peppermint oil demonstrated significant pigment inhibitory activity in CVO26 but lavender and cinnamon oils showed less intense pigment inhibitory activity.

On the basis of the preliminary anti-quorum sensing screening, two antibiotics (Doxycycline and Ceftazidime), three methanolic plant extracts (*Mangifera indica* (leaf), *Psoralea corylifolia* (seed) and *Trigonella foenum-graceum* (seed)) and two essential oils (clove and peppermint) were selected for further studies on the quorum sensing regulated virulence factors/traits of *Pseudomomas aeruginosa* PAO1 and or other related bacteria. At selected sub-MICs no significant growth inhibition was recorded over control.

The selected fractions of plant extracts, essential oils and antibiotics were firstly subjected to MIC determination against all test strains. Sub-MICs of screened

agents were selected for studies on QS regulated virulence factors/traits and biofilm formation.

Effect of antibiotics on quorum sensing regulated virulence factors/traits

The extent of inhibition of violacein was determined by the extraction of violacein pigment from CVO26 in the presence and absence of doxycycline. Doxycycline at sub-MICs (1-4 μg/ml) exhibited concentration-dependent pigment inhibitory activity and which ranged from 40.4-70% without any significant inhibition of growth.

The sub-MICs (0.5, 1, 2 and 4 μg/ml) of doxycycline showed a concentration-dependent effect on production of virulence factors of *P. aeruginosa* PAO1. Statistically significant percentage reduction ($p \leq 0.05$) in pyocyanin production, total protease activity and swarming motility was recorded at 1 and 2 μg/ml. Doxycycline at 4 μg/ml concentration caused maximum percentage decrease of 73.1% in swarming motility followed by pyocyanin production, chitinase, protease and elastase in *P. aeruginosa* PAO1. Similarly, a concentration dependent effect of doxycycline on EPS production was observed. The clinical strain *Pseudomonas aeruginosa* (PAF79) showed a MIC of 64 μg/ml against doxycycline. Therefore, a range of sub-MICs (4, 8, 16 and 32 μg/ml) were selected. A concentration dependent reduction in the above tested virulence factors was recorded.

Doxycycline at above tested concentrations significantly inhibited biofilm formation by PAO1. Maximum reduction (78.8%) in biofilm forming ability was recorded at highest sub-MIC (4 μg/ml) tested. Similarly, significant concentration ($p \leq 0.005$) dependent decrease in biofilm formation was also observed in PAF79 strain when grown in the presence sub-MICs of doxycycline.

The effect of antibiotic was also assessed against selected QS regulated virulence factors/traits of *Aeromomas hydrophila* WAF38. Highest tested concentration (8 μg/ml) was found effective against all the three traits tested and significant reduction in total protease activity (66.9%), EPS production (51%) and biofilm formation (71.5%) was recorded.

Effect of ceftazidime on quorum sensing regulated virulence factors/traits

In *Chromobacterium violaceum* CV026, a maximum of 74.6% inhibition in violacein production was observed at the concentration of 0.25 μg/ml of ceftazidime. No

significant reduction in growth of CVO26 was observed at all the tested concentrations.

The cell-free supernatant of ceftazidime-treated PAO1 exhibited a significant reduction in the azocasein-degrading proteolytic activity (55.7%), elastin-degrading elastase activity (62.8%), chitinase activity (63.8%) and pyocyanin production (61.1%) at 0.5 µg/ml concentration over untreated control. Similar concentration dependent decrease in the QS regulated virulence factors was also observed in the clinical strain of *P. aeruginosa* PAF79. Elastase, total protease activity, chitinase activity and pyocyanin production was reduced significantly ($p \leq 0.05$) only at 2 µg/ml.

A significant ($p \leq 0.005$) decrease in biofilm formation was also observed in test bacterial strains when grown in the presence of ceftazidime. In PAO1, significant reduction of 70% was observed at 0.5 µg/ml concentration. In a similar manner biofilm forming ability of PAF79 was reduced significantly at 1 and 2 µg/ml and a maximum of 65.3% reduction was recorded. EPS production and swarming motility that play a major role in biofilm formation were also inhibited significantly in a dose dependent manner in both the *P. aeruginosa* strains.

Concentration dependent decrease in the virulence factors (total protease and EPS production) was recorded in *A. hyrophila* WAF38. Most effective concentration was found to be 0.5 µg/ml as significant reduction in total protease activity (56.9%), EPS production (60.2%) and biofilm formation (65.2%) was recorded.

Effect of most active fraction of plant extracts on quorum sensing regulated virulence factors

Effect of methanolic extract of *Mangifera indica* (leaf)

The effect of methanolic fraction of *M. indica* (leaf) on QS interference activity in terms of reduced violacein production in CVO26 supplemented with pure AHL revealed maximum of 83.6% reduction in violacein at highest tested sub-MIC as compared to control. The decrease in violacein production was statistically significant at all tested concentrations except for the lowest concentration (100 µg/ml).

Methanolic extract of *M. indica* at highest tested concentration (800 µg/ml) caused reduction in elastase (76.2%), total protease (56%), pyocyanin (88.8%), and chitinase (55.3%). Similarly, effect of the extract on *P. aeruginosa* PAF79 was also

assessed at sub-MICs (125, 250, 500 and 1000 µg/ml). All tested concentrations demonstrated significant reduction in the activity of elastase (50.8-73.6%), total protease (57.5-82.9%), chitinase (61.1-76.9%) and swarming motility (45.8-70.3%).

Sub-MICs of *M. indica* (leaf) extract exhibited reduced biofilm formation in PAO1 and PAF79. PAO1 showed significantly decreased biofilm formation in the presence of sub-MICs of extract. In PAF79 biofilm formation was reduced considerably at 1000 µg/ml (74%), moderately at 500 µg/ml and non-significantly at 125 µg/ml .

The extract demonstrated significant dose dependent reduction in total protease activity and EPS production by *A. hydrophila* strain. The extract reduced biofilm formation maximally by 82.3% at 1000 µg/ml. At lower concentrations also the reduction was significant ranging from 56.1-76.1%.

Effect of methanol extract of *Psoralea corylifolia* (seed)

The extract of *P. corylifolia* exhibited concentration-dependent violacein inhibitory activity at all tested concentrations. Maximum reduction of 63.3% over control was observed at 600 µg/ml concentration of the extract.

Quorum sensing interference by methanol extract of *P. corylifolia* (seed) against *P. aeruginosa* strains showed consistent reduction in LasB elastolytic activity of PAO1 and PAF79 by 49.7 and 46.1 %, respectively. Similarly, total proteolytic assay was reduced by 50.5% in PAF79 and 43.5% in PAO1 at 1000 µg/ml. Pyocyanin production was reduced significantly at all concentrations in PAO1. However, in PAF79 pyocyanin production was reduced maximally to 57.8% over untreated control at 800 µg/ml. Chitinase activity in both the strains was impaired significantly upon treatment with sub-MICs of the extract.

A significant ($p \leq 0.005$) decrease in biofilm formation of *P. aeruginosa* strains was observed when grown in the presence of *P. corylifolia* extract. The plant extract effectively interfered with the production of EPS and swarming motility in PAO1 and PAF79.

The extract of *P. corylifolia* (100–800 µg/ml) effectively interfered with the QS regulated traits of *A. hydrophila* WAF38 and showed significant reduction in total protease activity and EPS production. Dose dependent reduction in biofilm formation

A. hydrophila WAF38 was observed. Maximum reduction of 50.8% ($p \leq 0.05$) was recorded at 800 µg/ml concentration of the extract.

Effects of methanol extract of *Trigonella foenum-graceum* (seed)

Interference of QS by methanol extract of *Trigonella foenum-graceum* at sub-MICs (125, 250, 500 and 1000 µg/ml) was determined using violacein assay in CVO26. The extract exhibited increased production of violacein pigment by CVO26 at all tested concentrations. Maximum of 74.2% ($p \leq 0.005$) increase in violacein production at 1000 µg/ml concentration was recorded.

The extract was further evaluated against QS regulated virulence factors in *P. aeruginosa* PAO1 and clinical strain PAF79. Statistically significant reduction ($p \leq 0.05$) in elastase, total protease, chitinase activity, pyocyanin production, EPS production and swarming motility was recorded at 1000 µg/ml.

Sub-MICs ranging from 125-1000 µg/ml were selected against clinical strain PAF79. Maximum inhibition in the activity of elastase (67.6%), protease (55%), chitinase (87%), pyocyanin production (82.1%) and swarming motility (62.5 %) was observed at 1000 µg/ml of extract. EPS production was reduced significantly at all tested concentration in PAF79 strain and maximum of 77.5 % decrease was observed at highest tested concentration of *T. foenum-graceum* extract.

T. foenum-graceum extract demonstrated 24.1-68.7 % decrease in the biofilm forming ability at sub-MICs tested. Correspondingly, significant concentration ($p \leq 0.005$) dependent decrease in biofilm formation was also observed in PAF79 strain when grown in the presence sub-MICs of extract. The effect of the extract was also assessed against virulence factors of *A. hydrophila* WAF38. Highest tested concentration (800 µg/ml) was found effective against all the three parameters tested and significant reduction in total protease activity (71.6%), EPS production (46.3%) and biofilm formation (76.9%) was recorded.

Effect of essential oils (clove and peppermint) on quorum sensing regulated virulence factors

Syzygium aromaticum (clove) oil inhibited violacein production upto 78.4% at maximum sub-MIC (0.12%) tested with little or no significant growth inhibition of *Chromobacterium violaceum* CVO26. Similarly, a concentration dependent decrease

in all the tested QS linked functions was evident in *Pseudomonas aeruginosa* PAO1 and PAF79. Maximum inhibition of pyocyanin (75%) over control at sub-MICs (1.6%) was recorded in PAO1. EPS production (77%) and swarming motility (80%) were also inhibited significantly in a concentration dependent manner. Further, clove oil demonstrated highest inhibition activity in pyocyanin production followed by swarming motility, total protease, elastase activity, EPS production and least in chitinase activity.The oil was also tested for its antibiofilm activity against at their respective sub-MICs. Significant ($p \leq 0.05$) decrease in biofilm formation was observed in test bacterial strains. The oil showed a maximum of 65% and 83.3% reduction in biofilm formation by PAO1 and PAF79 at respective highest tested sub-MICs.

Further, effect of sub-MICs of clove oil on virulence factor of *Aeromonas hydrophila* resulted in a significant decrease in total protease (57%), EPS production (71%) at highest tested sub-MIC value tested. Similarly, a concentration dependent decrease (35-66%) in biofilm formation was observed in the test bacteria when treated with oil at varying concentrations.

Anti-QS property of *Mentha piperita* (peppermint) oil was assessed for pigment inhibition in CVO26. The sub-MICs of peppermint oil exhibited concentration-dependent violacein inhibitory activity at all tested concentrations. The effect of sub-MICs of oil in reducing the production of QS-dependent virulence factors in PAO1 and PAF79 was investigated. Significant decrease in pyocyanin production (52.4-85.2%), EPS production (39.9-76.52%) and swarming motility (50.6-81.3%) of PAO1 was recorded at sub-MICs of the oil tested. Sub-MICs (0.75-3%) were found effective in inhibiting activity of elastase, total protease and chitinase to significant levels. Similar concentration dependent reduction was recorded for PAF79 treated with sub-MICs (0.2-1.6%) of peppermint oil. Maximum reduction of 87.3% was recorded in pyocyanin production followed by elastase activity, chitinase activity, swarming motility, EPS production and total protease activity at the highest tested concentration (3%) tested over control.

Anti-biofilm activity of the test oil was investigated against PAO1 and PAF79. Addition of respective highest sub-MICs of oil led to a dose dependent reduction in biofilm formation by upto 84% and 88.1% in PAO1 and PAF79, respectively.

The oil of peppermint effectively interfered with the QS system of *A. hydropila* WAF38 by significantly reducing total protease activity to the level of 24.5–71% ($p \leq 0.005$) and EPS production by 39-77.9%. Maximum decrease (74.8%) in biofilm formation at 0.8% v/v concentration of the oil was recorded.

Effect of essential oil major compounds (eugenol and menthol) on QS regulated virulence factors

Eugenol exhibited a concentration dependent decrease in QS regulated violacein production and statistically significant inhibition was recorded at all tested concentrations. Significant reduction ($p \leq 0.001$) in elastase (47-82%), total protease activity (44-87%) and pyocyanin production (45-85%) of PAO1 was recorded at tested sub-MICs of eugenol. Similarly, eugenol demonstrated significant inhibitory activity on total protease (61.1-91.3%), pyocycanin production (31.1-82.2%) an EPS production (31.3-69.8%) of PAF79 at sub-MICs tested. None of the concentrations tested could inhibit the swarming motility of PAO1 and PAF79 to statistically significant levels. However, biofilm formation in both the strains of *P. aeruginosa* and *A. hydrophila* WAF38 was reduced considerably upon treatment with sub-MICs of eugenol.

Eugenol demonstrated dose dependent reduction in the total protease activity and EPS produced by A. *hydrophila* WAF38 also. Total protease activity was reduced by 43.2-64.2% followed by EPS production by 43.6-69.9% at sub-MICs (0.15-1.5% v/v) of eugenol.

Menthol exhibited a concentration dependent decrease in QS regulated violacein production. Maximum reduction of 85% was recorded at 400 µg/ml concentration while lowest of 26% decrease over control was observed at 50 µg/ml menthol concentration. Effect of menthol on QS regulated virulence factors of *Pseudomomas aeruginosa* PAO1 and PAF79 revealed a concentration dependent decrease in all the functions. Decrease in total protease activity was highest (84.2%) followed by pyocyanin production (83.5%), elastase activity (78.7%), swarming motility (78%), EPS production (57.7%) and least in chitinase activity (54.6%). Similar dose dependent decrease was recorded in the virulence factors of PAF79.

In *Aeromonas hydrophila* WAF38, menthol inhibited total protease significantly (52.5%) at 200 µg/ml while at lower concentrations reduction observed was not statistically significant. EPS produced by untreated *A. hydrophila* WAF38 was lowered significantly (58.3-66.6%) at sub-MICs (50-200 µg/ml). Similarly, biofilm formation was also reduced ranging from 27.9-80% over untreated control at sub-MICs of menthol tested.

β-galactosidase assay

Effect of sub-MICs of test agents on β-galactosidase activity exhibited a concentration dependent decrease. Significant reduction in AHL levels in *E. coli* MG4/pKDT17 was recorded at respective sub-MICs. The reduction of β-galactosidase activity in *E. coli* MG4/pKDT17 shows that antibiotic, plant extracts, essential oil and phytocompounds mediated inhibition of *lasB* promoter activity involves LasR controlled transcription.

Anti-infective potential of test agents in *C. elegans* nematode model

The anti-infection potential of the sub-MIC of test agents was assessed using a liquid killing assay of *C. elegans* by PAO1. Complete (100%) mortality of the *P. aeruginosa* PAO1 preinfected *C. elegans* was observed within 72 h. However, *C. elegans* preinfected with PAO1 and treated with doxycycline (4 µg/ml) and ceftazidime (0.5 µg/ml) separately displayed enhanced survival rate of 55% and 61% respectively. Similarly, plant extracts of *M. indica*, *P. corylifolia* and *T. foenum-graceum* also displayed an enhanced survival rate of 72%, 58% and 48% respectively. Essential oils and their phytocompounds tested also increased the survival of the nematode significantly (58-71%) in comparison to *P. aeruginosa* infected nematode.

Phytochemical analysis of plant extracts

On the basis of promising QS interference activity of three plant extracts were subjected to determination of total phenolics contents in all fractions obtained. The total phenolic content in various fractions ranged from 32.3-538.2 mg GAE/g of dry extract. Futher major groups of phytocompounds were determined in active fractions by color test and infrared spectroscopy (IR) analysis.

***Mangifera indica* (leaf) extract**

Phytochemical analysis of *Mangifera indica* extracts revealed the presence of tannins, glycosides and phenolics as major groups of compounds. The total phenolic content

(mg GAE/g) of extract and various fractions determined by the Folin-Ciocalteu method showed highest polyphenolic content (538±3.4) in acetone fraction followed by methanol, ethyl acetate, benzene and least in petroleum ether fraction. Most active fraction was GC-MS analysis which revealed the presence 16 components using direct similarity search for *M. indica*. The compounds identified were 1,2,3-Benzenetriol (15.6%), Benzoic acid, 4-hydroxy (12.09%), n-Hexadecanoic acid (9.96%), 4H-Pyran-4-one, 2,3-dihydro-3,5-dihydroxy-6-methyl (8.48%) as evident from the GC-MS spectra. These numbers may be extended with the help of chemo metric techniques.

***Psoralea corylifolia* (seed) extract**

Phytochemical analysis of *P. corylifolia* (seed) revealed the presence of alkaloids, phenolics and tanins as major group of compounds. The total phenolic content of various fractions (mg/g of dry extract) was determined as gallic acid equivalent by the Folin-Ciocalteu method. Methanol fraction of seed contained 367.6±1.5 mg GAE / g of dry extracts followed by acetone, ethyl acetate, benzene and pertroleum ether fractions. A total of 21 chemical components were identified in seed extract by GC-MS analysis. These numbers may be extended with the help of chemo metric techniques. The major compounds identified were 9,12-Octadecadienoic acid (35.72%) followed by 4-[3,7-Dimethyl-3-vinyl-1,6-octadienyl]phenol (27.73%), Palmitic acid (23.12%), Myristic acid (1.050%), The remaining compounds were present in percentages of 0.1 to 0.5.

***Trigonella foenum-graceum* (seed) extract**

Phytochemical analysis of fractions revealed the presence of alkaloids, phenolics, tanins and glycosides as major group of compounds. The total phenolic content (mg/g) of *Trigonella foenum-graceum* various fractions showed highest polyphenolic content (199.8±2.3) in methanol fraction followed by acetone (132.3±1.5), petrol ether (146.4±6.3), benzene (77.7±0.65), ethyl acetate (65.4 ±1.9) and petroleum ether (56±1.1) fractions.A total of 18 chemical components were identified in leaf extract by GC-MS analysis. These numbers may be extended with the help of chemo metric techniques. The major compounds identified were 1,3,7-Trimethyl-3,7-dihydro-1h-purine-2,6-dione (40.82%) followed by Methyl 14-methylpentadecanoate (8.22%), Palmitic acid (6.41%), 1,2,3-Benzenetriol (6.13%), Linoleic acid, methyl ester

(5.58%) and Capric acid (4.2%). The remaining compounds were present in percentages of 2.01 to 0.1.

Phytochemical analysis of clove oil

Major ingredients of clove oil as revealed by GC–MS analysis is eugenol (74.32%), and other constituents identified were α-caryophyllene (4.05%), iso-caryophyllene (5.96%), caryophyllene oxide (2.41%), β-caryophyllene (4.92%), napthalene, 1,2,3,5,6,8a-hexahydro- 4,7-dimethyl-1-(1-methyl ethyl) (7.04%) and 1,6-Octadiene-ol-,3,7-dimethyl acetate (1.28%).

Phytochemical analysis of peppermint oil

Major ingredients of peppermint oil as revealed by GC– MS analysis are menthol (36.87%), and other constituents identified were menthone (16.44%), neoisomenthol (11.33%), isomenthone (10.47%), menthyl acetate (7.47%), 2-isopropyl-5-methylcyclohexanol (2.74%), piperitone (2.17%) and limonene (0.53%).

Conclusions

Following conclusions can be drawn from the data obtained in the present study:

- *Pseudomonas aeruginosa* isolated from clinical and hospital environment are resistant to commonly used antibiotics and have biofilm forming capacity and could produce one or more quorum sensing signals (AHL) molecules. Both clinical and environmental isolates have active QS systems.
- QS regulated virulence factors in *Pseudomonas aeruginosa* and *Aeromonas hydrophila* were inhibited in a concentration dependent manner by antibiotics (doxycycline and ceftazidime). Similar reduction in the biofilm and its associated factors like exopolysaccharide (EPS) production and swarming motility was also significantly inhibited.
- On the basis of the preliminary screening studies, methanol extract of selected plants (*Mangifera indica, Psoralea corylifolia* and *Trigonella foenum-graceum*) significantly inhibited virulence factors like elastase, total protease, chtinase, pyocyanin production, EPS, swarming motility and biofilm formation to varying levels in a concentration dependent manner.
- Clove and peppermint oil inhibited biofilm and QS regulated virulence traits of PAO1, PAF79 and *A. hyrophila* WAF38. The efficacy of the oils was found

mainly attributed to their essential active constituents, eugenol and menthol. These active compounds also significantly inhibited virulence traits and biofilm formation in the test strains *in vitro*.

- β-galactosidase assay revealed that all the test agents significantly reduced AHL level sat respective sub-MICs. Reduced AHL levels are indicative of impaired *las*-controlled transcription, therefore reduced expression of *lasB* gene.
- The efficacy of anti-QS active antibiotics, plant extracts, essential oils and phytocompounds in *C. elegans* model highlights their therapeutic potential in combating *Pseudomonas* infection.
- Future research in this direction requires understanding the actual mechanism of QS inhibition at molecular level. Careful investigation in identifying the key compounds responsible for such broad spectrum antivirulence and antibiofilm activity should be isolated from plant extracts (*M. Indica*, *Psoralea corylifolia* and *T. foenum-graceum*) and role of other interacting molecules may be explored. Combinational approach of anti-QS agents from plants with antibiotics needs to be explored for possible strategy to combat pathogens.

Publications

Research papers published

1. **Fohad Mabood Husain**, Iqbal Ahmad, Mohammad Asif and Qudsia Tahseen. Influence of clove oil on certain quorum sensing regulated functions and biofilm of *Pseudomonas aeruginosa* and *Aeromonas hydrophila*. Journal of Biosciences **(Accepted).** Impact factor **1.759**
2. **Fohad Mabood Husain**, Iqbal Ahmad (2013). Quorum sensing inhibitors from natural products as potential novel anti-infective drug. Drugs of the Future **(Accepted).**
3. **Fohad Mabood Husain** and Iqbal Ahmad (2013). Doxycycline interferes with quorum sensing mediated virulence factors and biofilm formation in Gram negative bacteria. World Journal of Microbiology and Biotechnology. 29: 949-957. Impact factor **1.532**

Book chapters published

1. Iqbal Ahmad, **Fohad Mabood Husain**, Meenu Maheshwari amd Maryam Zahin (2013). Medicinal plants and phytocompounds : A potential source of novel antibiofilm agents. In: *Antibiofilm Agents: From Diagnosis to Treatment and Prevention*. Eds. Kendra P.Rumbaugh and Iqbal Ahmad. Springer **(Accepted)**
2. Iqbal Ahmad, Mohd. Sajjad Ahmad Khan, **Fohad Mabood Husain**, Maryam Zahin and Mahipal Singh (2011). Bacterial quorum sensing and its interference: Methods and significance. In: *Microbes and Microbial Technology: Agricultural and Environmental Applications*. Eds. Iqbal Ahmad, Farah Ahmad and John Pichtel. Springer, USA pp. 127-162.

Research papers communicated

1. **Fohad Mabood Husain** and Iqbal Ahmad (2013). Broad spectrum anti-quorum sensing and antibiofilm activity of *Mentha piperita* oil and its major phytoconstituent menthol against negative bacterial pathogens. Future Microbiology **(Impact factor 4.0).**
2. **Fohad Mabood Husain** and Iqbal Ahmad (2013). Anti-quorum sensing and biofilm inhibitory activity of *Mangifera indica* (leaf) extract. Journal of Applied Microbiology **(Impact factor 2.1).**

Influence of clove oil on certain quorum-sensing-regulated functions and biofilm of *Pseudomonas aeruginosa* and *Aeromonas hydrophila*

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Quorum sensing (QS) plays an important role in virulence, biofilm formation and survival of many pathogenic bacteria including *Pseudomonas aeruginosa*. This signalling pathway is considered as novel and promising target for anti-infective agents. In the present investigation, effect of the Sub-MICs of clove oil on QS regulated virulence factors and biofilm formation was evaluated against *P. aeruginosa* PAO1 and *Aeromonas hydrophila* WAF-38 strain. Sub-inhibitory concentrations of the clove oil demonstrated statistically significant reduction of *las*- and *rhl*-regulated virulence factors such as LasB, total protease, chitinase and pyocyanin production, swimming motility and exopolysaccharide production. The biofilm forming capability of PAO1 and *A. hydrophila* WAF-38 was also reduced in a concentration-dependent manner at all tested sub-MIC values. Further, the PAO1-preinfected *Caenorhabditis elegans* displayed an enhanced survival when treated with 1.6% v/v of clove oil. The above findings highlight the promising anti-QS-dependent therapeutic function of clove oil against *P. aeruginosa*.

[Husain FM, Ahmad I, Asif M and Tahseen Q 2013 Influence of clove oil on certain quorum-sensing-regulated functions and biofilm of *Pseudomonas aeruginosa* and *Aeromonas hydrophila*. *J. Biosci.* **38** 835–844] **DOI** 10.1007/s12038-013-9385-9

1. Introduction

Multi-drug resistant (MDR) bacteria have become a global health problem resulting in mortality of millions of people annually due to infections. Since the progress in the discovery of new antibacterial drugs with novel mode of actions is poor globally, alternative approaches to combat these resistant strains are the need of the hour (Rasmussen *et al.* 2005; Ahmad *et al.* 2008). It is important to emphasize that of all the infectious diseases, at least 65% are associated with the bacterial communities which proliferate by forming biofilms (Lewis 2007). Biofilm formation by many pathogens is closely linked to a density-dependent cell–cell communication known as quorum sensing (QS), in which small diffusible signalling molecules globally regulate expression of various genes including virulence genes (Fuqua *et al.* 2001; Rumbaugh *et al.* 2009). *Pseudomonas aeruginosa* is an opportunistic pathogen in which the role of QS-regulated virulence factors and biofilm is well studied in disease development (Rutherford and Bassler 2012). It is well known that *P. aeruginosa* employs the *las* and *rhl* AHL-based QS systems. LasR is a transcriptional regulator protein that recognizes its ligand (3-oxo-C12-HSL) and triggers the expression of LasB elastase, toxin production and biofilm formation (Pesci *et al.* 1997; Rumbaugh 2004). RhlR is the other transcriptional regulator protein which responds to *N*-butanoyl-L-homoserine lactone (C4-HSL) and regulates pyocyanin production (Williams 2007). Recently, a new QS signal, IQS, has been identified which is tightly controlled by *las* under normal culture conditions but is also activated by phosphate limitation, a common stressor that bacteria encounter during infections, indicating a more complex QS system in bacteria (Lee *et al.* 2013). *Aeromonas hydrophila* has also been described as an opportunistic pathogen that produces *N*-butanoyl-L-homoserine lactone (C4-HSL) as the principal AHL (Swift *et al.* 1997). The AHL-dependent QS plays a crucial role in the virulence and biofilm development of this pathogen (Williams, 2007). Since QS plays an important role in virulence and survival of *P. aeruginosa* and other pathogenic bacteria, this signalling pathway is a novel and potential target for anti-infective agents (Hentzer and Givskov 2003;

Keywords. Biofilm; *C. elegans*; clove oil; quorum-sensing inhibition

Published online: 6 November 2013

World J Microbiol Biotechnol
DOI 10.1007/s11274-013-1252-1

ORIGINAL PAPER

Doxycycline interferes with quorum sensing-mediated virulence factors and biofilm formation in Gram-negative bacteria

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Received: 13 July 2012 / Accepted: 2 January 2013

Abstract Inhibition of quorum sensing (QS)-regulated virulence factors including biofilm is a recognized anti-pathogenic drug target. The search for safe and effective anti-QS agents is expected to be useful to combat diseases caused by multidrug-resistant bacteria. In this study, effect of a commonly used antibiotic, doxycycline on QS was evaluated using sensor strains of *Chromobacterium violaceum* (ATCC 12472 and CVO26) and *Pseudomonas aeruginosa* PAO1. Sub-MICs of doxycycline reduced QS-controlled violacein production in *C. violaceum* to a significant degree (70 %) and showed a significant reduction of LasB elastase (67.2 %), pyocyanin (69.1 %), chitinase (69.8 %) and protease (65 %) production and swarming motility (74 %) in *P. aeruginosa* PAO1 over untreated controls. Similar results were also recorded against a clinical strain of *P. aeruginosa* (PAF-79). Interestingly, doxycycline at respective sub-MICs (4 and 32 μg ml^{-1}) significantly reduced the biofilm-forming capability and exopolysaccharide production in both the strains of *P. aeruginosa* (PAO1 and PAF-79) over untreated controls. The results of this study highlight the multiple actions of doxycycline against QS-linked traits/virulence factors and its potential to attenuate virulence of *P. aeruginosa*.

Keywords Quorum sensing · *Chromobacterium violaceum* · *Pseudomonas aeruginosa* · Virulence factors · Biofilm

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Introduction

Quorum sensing (QS) is defined as the ability to detect extracellular, small molecule signals and to alter gene expression in response to bacterial population densities (Camara et al. 2002). Elements of the QS regulatory circuit of bacteria are now known to serve a wide variety of functions beyond a simple estimate of cell density. The basic principles behind QS signal-mediated gene expression in both Gram-positive and Gram-negative bacteria are shared (Williams 2007), but the molecular mechanisms and signal molecules differ. Gram-negative signaling systems are based on N-acylhomoserine lactone (AHL) signal molecules and AHL-based communications are the most intensively studied examples of QS (Fuqua and Greenberg 2002). AHL production is considered indicative of the presence of functional QS regulatory circuits (Bjarnsholt and Givskov 2008).

To overcome the growing problem of antibiotic resistance, an anti-pathogenic approach has been recently considered as a viable alternative. Many pathogenic bacteria employ quorum sensing to regulate their pathogenicity and virulence factor production. Inhibition of QS seems an attractive target (Antunes et al. 2010). *Pseudomonas aeruginosa* is an opportunistic pathogen and a major cause of nosocomial diseases and mortality in immunocompromised patients and particularly in patients with cystic fibrosis, diffused panbronchitis, and pulmonary deficiencies (Driscoll et al. 2007; Mesaros et al. 2007). The *las* and *rhl* quorum-sensing systems regulate the production of several extracellular virulence factors, including elastase, exoproteases, siderophores, exotoxins, rhamnolipid and several secondary metabolites, and participate in the development of biofilms (Davies et al. 1998; Van Delden et al. 1998; Hentzer et al. 2003). However, some reports have indicated biofilm formation by QS-deficient *P. aeruginosa* (Schaber et al. 2007). Owing to the importance of quorum sensing during bacterial pathogenesis,

Published online: 09 January 2013

Drugs of the Future 2013, 38(10): 691-706

CCC: 0377-8282/2013
DOI: 10.1358/dof.2013.38.10.2075393

QUORUM SENSING INHIBITORS FROM NATURAL PRODUCTS AS POTENTIAL NOVEL ANTIINFECTIVE AGENTS

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SUMMARY

Natural products are known to contribute significantly in primary healthcare and the development of drugs for various ailments, including infectious diseases. In recent decades, the increasing problem of the emergence of multidrug resistance in bacteria and the slow progress in the discovery and registration of new antibiotics has necessitated the search for new antiinfective drugs with novel modes of action and/or the use of alternative strategies. Interference with quorum sensing of bacterial pathogens to attenuate their virulence is considered a novel antiinfective drug target. In the last decade, there has been interest among the scientific community to explore broad-spectrum, nontoxic and stable quorum sensing inhibitors. Efforts are being made in this direction to screen and evaluate natural products from medicinal plants and other sources. The present review indicates that a large number of medicinal plant extracts, phytocompounds and other natural products inhibit quorum sensing regulated functions in different bacterial pathogens. It appears that quorum sensing inhibitors of plant origin, e.g., garlic and derivatives, have shown promising efficacy in animal models and could be developed as antiinfective drugs. Numerous quorum sensing inhibitors have shown synergy with antibiotics and could be further explored to obtain effective combination formulations combating drug-resistant bacterial pathogens.

Key words: Bacterial quorum sensing – Plant extracts – Natural products – Medicinal plants – Phytocompounds – Quorum sensing inhibitors

INTRODUCTION

When the body's normal defense cannot overcome a disease, it is often treated with chemotherapeutic agents such as antibiotics. Antibiotics have reduced the death rate from infectious diseases. However, excessive and indiscriminate use of antibiotics has also contributed to the rapid evolution of drug resistance among clinically significant bacterial species (1). There has been an increase in the reports of treatment complications and failures due to multidrug resistance in all human pathogens. This has necessitated the search for alternative strategies to combat bacterial infections (2, 3). Recent development in genomics and cell-to-cell communication has provided novel drug targets.

Quorum sensing is an intercellular signaling and gene regulatory mechanism used by bacteria to respond to their population density. It was first observed in *Vibrio fischeri*, a marine symbiotic bacterium, in controlling luminescence. Quorum sensing has been identified in bacteria such as *Chromobacterium violaceum*, *Pseudomonas aeruginosa*, *Enterobacter agglomerans*, *Burkholderia cepacia*, *Aeromonas hydrophila*, *Staphylococcus aureus* and many other bacteria for the control of diverse functions, including virulence factors (4). The quorum sensing process involves the generation of a signal molecule, accumulation of the signal molecule in medium to certain threshold concentrations, recognition of the signal molecule by a receptor and expression of a large array of genes in response to the concentration of the signal–receptor complex (5). Quorum sensing is known to overcome the host defense barriers by affecting the human transcriptional programs, detecting the human cytokines and stress hormones, and capture when the host is most vulnerable (3). Quorum sensing signals can transgress the interspecies and inter kingdom barriers (6).

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